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Protein levels of genes encoded on chromosome 21 in fetal Down Syndrome brain (Part V): Overexpression of phosphatidyl-inositol-glycan class P protein (DSCR5)

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Summary. Down Syndrome (DS, trisomy 21) is the most common genetic cause of mental retardation. The completed sequencing of genes encoded on chromosome 21 provides excellent basic information, however the molecular mechanisms leading to the phenotype of DS remain to be elucidated. Although overexpression of chromosome 21 encoded genes has been documented information at the protein expression level is mandatory as it is the proteins that carry out function. We therefore decided to evaluated expression level of seven proteins whose genes are encoded on chromosome 21: DSCR4, DSCR5, DSCR6; KIR4.2, GIRK2, KCNE1 and KCNE2 in fetal cortex brain of DS and controls at the early second trimester of pregnancy by Western blotting. β-actin and neuron specific enolase (NSE) were used to normalise cell loss and neuronal loss. DSCR5 (PIG-P), a component of glycosylphosphatidylinositol-*N*-acetylglucosaminyltransferase (GPI-GnT), was overexpressed about twofold, even when levels were normalised with NSE. DSCR6 was overexpressed in addition but when normalised versus NSE, levels were comparable to controls. DSCR4 was not detectable in fetal brain. Potassium channels KIR4.2 and GIRK2 were comparable between DS and controls, whereas KCNE1 and KCNE2 were not detectable. Quantification of these proteins encoded on chromosome 21 revealed that not all gene products of the DS critical region are overexpressed in DS brain early in life, indicating that the DS phenotype cannot be simply explained by the gene dosage effect hypothesis. Overexpression of PIG-P (DSCR5) may lead to or represent impaired glycosylphosphatidylinositol-*N*-acetylglucosaminyltransferase mediated posttranslational modifications and subsequent anchoring of proteins to the plasma membrane.

Keywords: DSCR4 – DSCR5 (PIG-P) – DSCR6 – KIR4.2 – GIRK2 – KCNE1 – KCNE2 – Fetal brain – Protein expression – Immunoblotting

Introduction

Down Syndrome is the most frequent genetic disorder with mental retardation (1:700 live births) caused by trisomy 21. Individuals with DS show a series of neuropathological features, including reduction of brain size, abnormal neuronal migration, differentiation and abnormal dendritic arborization (Epstein, 1995; Capone, 2001). According to a widely accepted concept, the gene dosage hypothesis is used for the explanation for the development of the DS phenotype. This hypothesis has been, however challenged by several groups including ours. Sequencing of genes encoded on chromosome 21 provided an excellent opportunity to determine the transcriptome and indeed a series of these genes were upregulated in fetal DS at the mRNA level. No definitive conclusion, however, can be drawn from the transcriptome as RNA levels do not regularly correspond to protein levels and moreover, posttranscriptional modifications have to be taken into account (Engidawork et al., 2003; Mao et al., 2003; Antonarakis, 2001).

In some cases, DS results from triplication of only a part of chromosome 21 (partial trisomy 21). This region is called Down Syndrome critical region (DSCR) and is supposed to encode genes responsible for the main features of DS (Ohira et al., 1996; Delabar et al., 1993). We therefore analyzed expression of seven gene products from this chromosomal region: DSCR4, DSCR5, DSCR6, KIR4.2, GIRK2, KCNE1 and KCNE2 (Fig. 1) as a part of a comprehensive study systematically quantifying gene products from chromosome 21.

DCRB (gene: DSCR4) is a novel protein and molecular and biological function remains still to be elucidated. The amino acid sequence of this protein showed no significant homology to any known protein, although Northern blot analysis demonstrated that DCRB is mainly expressed in the placenta (Nakamura et al., 1997).

PIG-P (gene: DSCR5) is a member of the GPI-GnT, the complex of enzymes realizing the first step in the

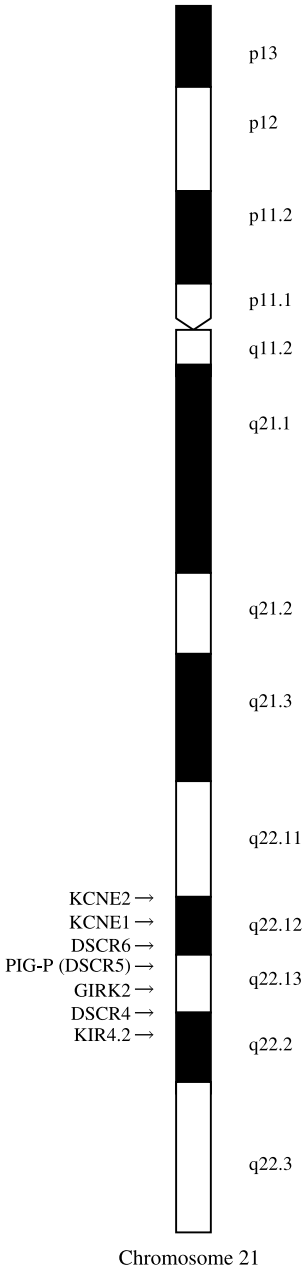

Fig. 1. Schematic drawing of the Giemsa banding (G-bands) of human chromosome 21. Arrows indicate seven gene products encoded on chromosome 21, examined in this study

glycosylphosphatidylinositol (GPI) anchor mode of protein binding to the plasma membrane (Watanabe et al., 2000). This protein consists of two N-terminal hydrophobic regions, one C-terminal hydrophilic region, two putative transmembrane regions, and is localized in the endoplasmatic reticulum (Watanabe et al., 2000). In humans DSCR5 has been detected at the mRNA level in several tissues including fetal brain at a low level (Shibuya et al., 2000).

DSCR6 is a novel protein with unknown function, and neither the sequence-similarity search by BLASTP nor motif analysis by SMART detected significant homologous proteins and motifs and PSORTII predicted a nuclear localization. Its expression is detectable using Multiple Tissue cDNA panels in fetal kidney and fetal brain at high levels, suggesting a role in early embryogenesis (Shibuya et al., 2000).

KIR4.2 (KIR1.3) is a potassium (K^+) channel belonging to the family of inward rectifier K^+ (Kir) channels, playing key roles in controlling membrane potential, cellular excitability and K^+ fluxes (Pearson et al., 1999). KIR4.2 homomeric channels possess an intrinsic sensitivity to intracellular acidification, and KIR4.2 also forms novel heteromeric channels with another Kir family member, KIR5.1 (Pessia et al., 2001). This channel showed tissue-specific expression when analyzed by Northern blots (Shuck et al., 1997). It is expressed in kidney and lung during human development and in several adult tissues including kidney and brain (Gosset et al., 1997). Week signals were detected by *in situ* hybridization in P30 mouse fetal brain. A specific band was obtained after PCR amplification of human fetal brain cDNAs but it may be produced in small quantities at the stage of 20 weeks of gestation (Thiery et al., 2003).

GIRK (also known as KIR3) channels are coupled to and regulated by neurotransmitter action and are part of a superfamily of Kir channels (Brown et al., 1990; Reimann et al., 1999). In general, neuronal GIRK are involved in the regulation of the excitability of neurons and may contribute to the resting potential (Mark et al., 2000). Recent data revealed that GIRK channels exist *in vivo* both as homotetramers and heterotetramers composed of several subunit combinations (Kofuji et al., 1995; Krapivinsky et al., 1995) and that functional GIRK channels are composed of GIRK2 and GIRK3, the most abundant subunits found in the mammalian brain (Jelacic et al., 2000). Strong signals in the reticular formation, in the cerebellar hemispheres and in the vermis were detected in the human fetal brain at the stage of 17 weeks of gestation by *in situ* hybridization (Thiery et al., 2003).

KCNE1 (minK channel, Isk) is the modulatory subunit of the slowly activating K^+ channel formed in association with KCNQ1 (also named KvLQT1) (Barhanin et al., 1996). Mutations of KCNE1/KCNQ1 lead to the Jervell and Lange-Nielsen syndrome, a disorder characterized by deafness and cardiac long QT syndrome (Tyson et al., 1997). KCNE1 plays a major role in the regulation of normal cardiac excitability and function of the inner ear

(Ehmke, 2002). At the mRNA level it was not detected in brain (Tinel et al., 2000).

KCNE2 is also a modulator subunit that associates with KCNQ2 and/or KCNQ3, two other six-transmembrane domain potassium channels. By *in situ* hybridization in rat brain, neuronal expression of KCNE2 is observed in several brain regions including cerebellum, neocortex, and hypothalamus. It was also high expressed in human brain at the mRNA level (Tinel et al., 2000).

Quantification of these proteins whose genes are encoded in the Down Syndrome critical region addressed the gene dosage effect and identified two novel proteins, PIG-P (DSCR5) and DSCR6 protein as possible pathogenetic factors for development of the DS phenotype.

Materials and methods

Fetal brain samples

Fetal brain tissues (cerebral cortex) of DS (8 females with 19.4 ± 1.1 weeks of gestational age) and controls (6 females with 19.1 ± 1.6 weeks of gestational age) were used in this study. Brain samples were obtained from Drs. Mara Dierssen, Genes and Disease Program, Genomic Regulation Center, Passeig Marítim 37–49, E-08003 Barcelona, Spain and JC Farreras, Unidad de Patología, Corporació Sanitaria Parc Tauli, Sabadell, Barcelona, Spain.

All samples had a postmortem time of less than 6 hours, and were stored at $-70°C$ with a freezing chain never interrupted until use.

Antibodies

The antibodies for DSCR4, DSCR5, DSCR6, KCNE1 and KCNE2 (goat polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, California, USA), Kir4.2 and GIRK2 (rabbit polyclonal antibodies, Alomone labs, Jerusalem, Israel), β-actin (mouse monoclonal antibody, IgG2a, Sigma, St.Louis, Missouri, USA) and neuron specific enolase (rabbit polyclonal antibody, Chemicon, Hampshire, UK) were purchased.

Western blotting

Fetal brain tissues ground under liquid nitrogen were homogenized in lysis buffer 1% SDS containing protease inhibitor cocktail tablets (Roche, Grenzach-Wyhlen, Germany), incubated 10 minutes at 37°C, boiled 10 minutes at 95°C and centrifuged at $8{,}000 \times g$ for 10 minutes. The BCA protein assay kit (Pierce, Rockford, Illinois, USA) was applied to determine the concentration of protein in the supernatant. Samples (10 μg) were mixed with the sample buffer (100 mM Tris-HCl, 2% SDS, 1% 2-mercaptoethanol, 2% glycerol, 0.01% bromophenol blue, pH 7.6), incubated at 95°C for 15 minutes and loaded onto a 12.5% ExcelGel SDS homogenous gel (Amersham Pharmacia Biotech, Uppsala, Sweden). Electrophoresis was performed with Multiphor II Electrophoresis System (Amersham Pharmacia Biotech, Uppsala, Sweden). Proteins separated on the gel were transferred onto PVDF membrane (Millipore, Billerica, Massachusetts, USA) and membranes were incubated in blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 and 2% non-fat 1 dry milk). Membranes were incubated for 2 hours at room temperature with diluted primary antibodies (1:500 for DSCR4, DSCR5, KCNE1, KCNE2 and GIRK2; 1:700 for DSCR6; 1:200 for KIR4.2; 1:5000 for β-actin and 1:2000 for NSE). After 3 times washing for 15 minutes with blocking buffer, membranes were probed with secondary antibodies (bovine anti-goat for DSCR4 (1:1000), DSCR5 (1:1000), DSCR6 (1:1400), KCNE1 (1:1000), KCNE2 (1:1000); goat anti-rabbit IgG (H + L) for KIR4.2 (1:400), GIRK2 (1:1000) and NSE (1:4000), and goat anti-mouse IgG2a for β-actin (1:5000) coupled to horseradish peroxidase (Southern Biotechnology Associates, Inc., Birmingham, Alabama, USA)) for 1 hour. Membranes were washed 3 times for 15 minutes and developed with the Western Lightning™ chemiluminescence reagents (PerkinElmer Life Sciences, Inc., Boston, Massachusetts, USA), (Cheon et al., 2003).

Statistics

The density of immunoreactive bands was measured by RFLPscan version 2.1 software program (Scanalytics, Fairfax, Virginia, USA). Between group differences were calculated by non-parametric Mann-Whitney U test using GraphPad Instat2 program and the level of significance was considered at $P < 0.05$.

Results

We evaluated expression levels of seven proteins encoded on chromosome 21, DSCR4, DSCR5, DSCR6, KIR4.2, GIRK2, KCNE1 and KCNE2 in fetal brain with DS compared to controls by Western blot analysis.

Two proteins, β-actin and NSE, were used as reference proteins for total cell- and neuronal density and their expression levels were comparable in controls and DS (Fig. 2).

We observed a single band at 40 kDa with the antibody against PIG-P/DSCR5, which was significantly about twofold overexpressed ($P < 0.05$) in DS which was also observed when normalised with NSE. When normalisation was calculated with β-actin ($p = 0.07$) PIG-P levels in DS were comparable to controls (Fig. 2, 3A, B).

We detected a single DSCR6-immunoreactive band at 37 kDa that was also significantly overexpressed in DS. However, when DSCR6 protein levels were corrected with either NSE or β-actin, levels were comparable between DS and control (Fig. 2, 3A, B).

No immunoreactive band was detected using the DSCR4 commercially available antibody.

Antibody against KIR4.2 recognized one band at 35 kDa and a high molecular mass band around 140 kDa in fetal brain, and statistical evaluation did not show any difference between DS and controls for both bands (Fig. 2, 3A, B). Different bands may represent isoforms, splicing variants or posttranslational modifications and several bands for this channel have been reported in hepatocytes (Hill et al., 2002).

A single band was observed with the GIRK2 antibody at 45 kDa, and no significant difference was found between DS and controls in fetal brain (Fig. 2, 3A, B).

No immunoreactive band was detected using commercially available antibodies against KCNE1 and KCNE2.

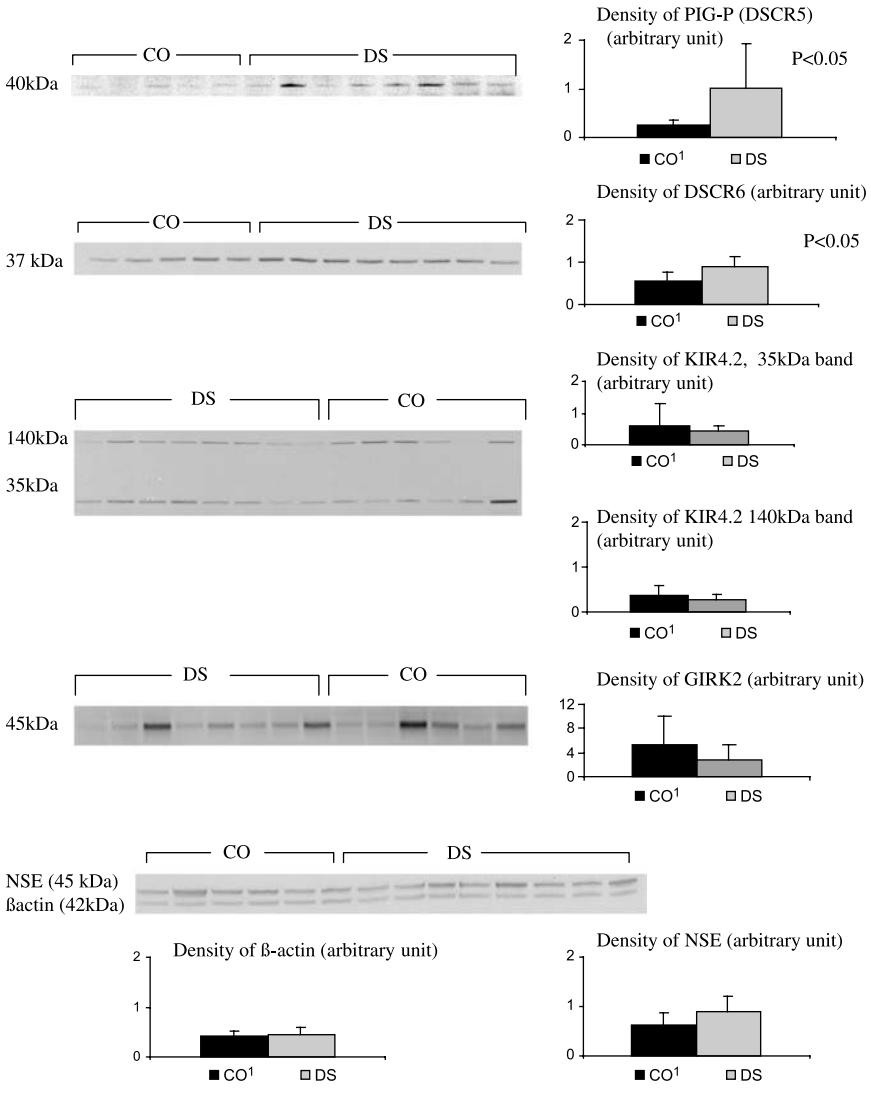

Fig. 2. Western blot analysis for four proteins whose genes are encoded on chromosome 21, β-actin and NSE in cerebral cortex from fetal brain with DS and controls. Denatured proteins (10 μg) were loaded, separated on a homogeneous gel and transferred onto PVDF membrane. As described in 'materials and methods', the membranes were incubated with primary and secondary antibodies, and immunoreactive bands (PIG-P(DSCR5), 40 kDa; DSCR6, 37 kDa; KIR4.2, 140 kDa and 35 kDa; GIRK2, 45 kDa; β-actin, 42 kDa; NSE, 45 kDa) were detected using chemiluminescence reagents. The density of detected bands was measured and calculated by non-parametric Mann-Whitney U test, and the level of significance was considered at $P < 0.05$. Results are presented as mean $\pm$ standard deviation

Expressional differences within DS or the control group may be representing allelic differences.

Discussion

The main finding of the study is to show that not all gene products from the Down Syndrome critical region (DSCR) are overexpressed as predicted by the gene dosage hypothesis as well as the observation that the DSCR5 and DSCR6 gene products PIG-P and DSCR6 protein are overexpressed in fetal DS brain. Increased expression of PIG-P, an integral component of the transferase GPI-GnT, may be reflecting or leading to impairment of this transferase system transfering N-acetylglucosamine from UDP-N-acetylglucosamine to phosphatidylinositol resulting into production of glycosylphosphatidylinositol (GPI), by titrating out other subunits (as e.g. PIG-A,GPI1) thus changing the required stoichiometry.

GPI, a complex glycolipid, mediates anchoring of more than 100 different proteins including cell surface enzymes, receptors, adhesion molecules etc., to the plasma membrane (Kinoshita et al., 1995) by C-terminal modification of proteins with a GPI attachment signal peptide (Ikezawa, 2002). This process of glycolipidization is a common posttranslation modification in eurkaryotes (Ferguson, 1999; Herscovics et al., 1993; Schultz et al., 1998; Udenfriend et al., 1995) and impaired GPI anchoring is detrimental for embryogenesis (Nozaki et al., 1999) and thus may play a role for the development of the brain deficit in DS. The increase of PIG-P cannot be assigned to possible changes of neuronal density in fetal DS as NSE levels were comparable between groups and when PIG-P

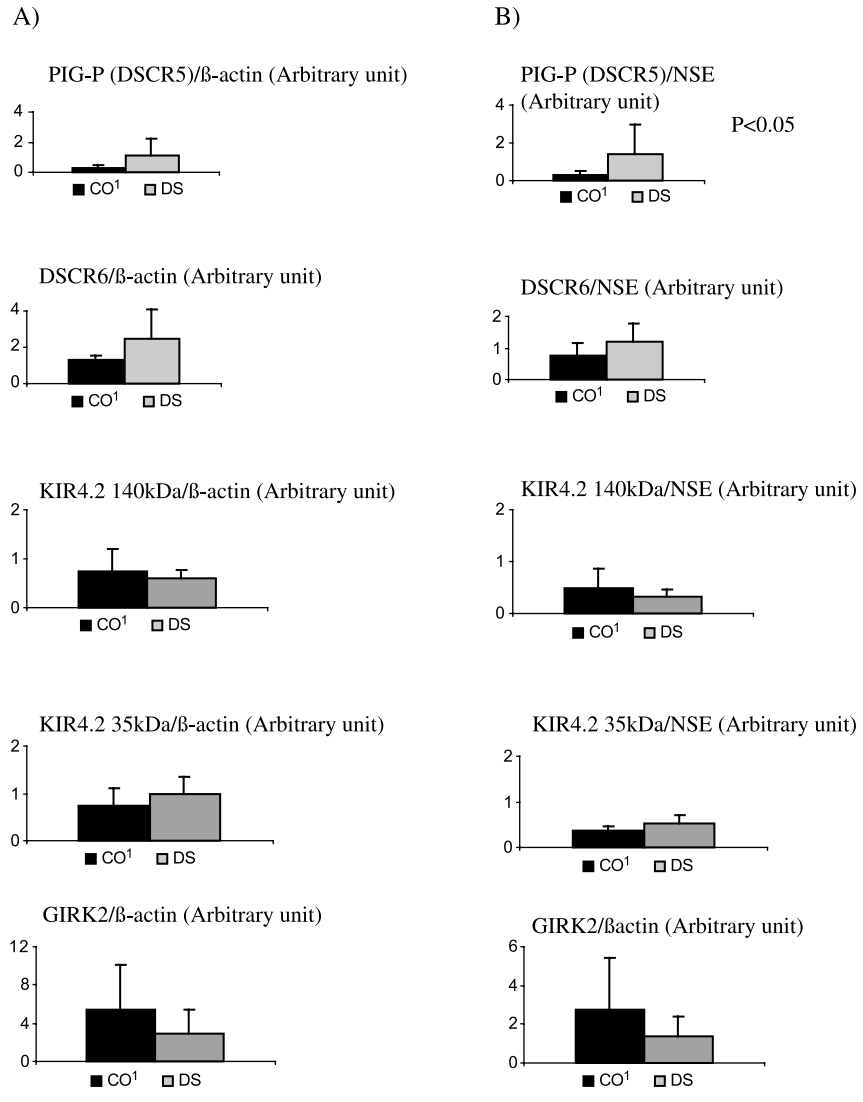

Fig. 3. Expression levels of four proteins (mean ± standard deviation) normalised with those of β-actin (**A**) or NSE (**B**). The density of immunoreactive band for each protein was normalised with that of β-actin or NSE, used as reference proteins. Data were analysed by non-parametric Mann-Whitney U test, and the level of significance was considered at $P < 0.05$

was normalized versus NSE, PIG-P levels still remained elevated twofold. Normalization of PIG-P versus the housekeeping protein β-actin still resulted into an increase (Fig. 3A) of PIG-P in fetal DS brain without reaching statistical significance, however.

DSCR6 protein levels were significantly increased in fetal DS; this finding is relativated when DSCR6 protein levels were normalized versus both, NSE and β-actin: corrected DSCR6 levels remained elevated in fetal DS brain, without reaching statistical significant as above.

As stated in the Introduction nothing is known about functions of DSCR6 and no functional domains were described or computed from structure and only its nuclear localization was proposed (Shibuya et al., 2000). It has never been described at the protein level (SWISSPROT P57055, DSR6_HUMAN) and is therefore a predicted protein that we now identify as an existing protein in human brain. Increased levels in fetal DS cortex may point to a putative function in the development of the brain deficit in DS.

The findings of two increased chromosome 21 gene products in fetal DS from above can be seen as specific as two other chromosome 21 gene products, KIR4.2 and GIRK2, showed comparable levels between groups. DSCR4, KCNE1 and KCNE2 were not detectable using immunoblotting. DSCR4 may not have been detected as it seems unlikely that this gene represents a protein coding transcript (Toyoda et al., 2002). KCNE1 was not observed in our system as also KCNE1 mRNA levels were not found in human brain (Tinel et al., 2000). Finally, KCNE2 was found at the mRNA level in adult human brain (Tinel et al., 2000) and it may well be absent in the fetal period and thus may explain our findings at the protein level.

In conclusion, we observed aberrant expression of PIG-P, the gene product of DSCR5 and DSCR6 protein, from the Down Syndrome critical region. Aberrant PIG-P may be representing or leading to impaired GPI mediated anchoring of proteins into the plasma membrane with the possible consequence of modulating brain development. On the other hand, we have shown the existence and increase of a predicted protein DSCR6 in human brain and a tentative involvement in DS pathogenesis. The fact that not all proteins encoded in the DS critical region are increased challenges the gene dosage hypothesis but it may be due to the fact that aberrant expression of some proteins may not be compatible with life and are secondarily downregulated to normal levels as it is the case for h2-calponin (Kuromitsu et al., 1997).

We are continuing to complete quantification of proteins whose genes are encoded on chromosome 21 in fetal brain in order to obtain a comprehensive expressional protein pattern in trisomy 21.

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