

Short Communication

Gene Expression Profile in Prion Protein-Deficient Fibroblasts in Culture

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To investigate the physiological function of the cellular isoform of prion protein (PrP^C), the gene expression profile was studied by analyzing a cDNA expression array containing 597 clones of various functional classes in two distinct skin fibroblast cell lines designated SFK and SFH, established from PrP-deficient (PrP^{-/-}) mice and PrP^{+/+} mice, respectively. The cells were incubated in the culture medium with or without inclusion of basic fibroblast growth factor (bFGF). When SFK cells were compared with SFH cells in untreated conditions, the expression of 15 genes, including those essential for cell proliferation and adhesion, was reduced, whereas the expression of 27 genes, including those involved in the insulin-like growth factor-I (IGF-I) signaling pathway, was elevated. Northern blot analysis verified a significant down-regulation of the receptor tyrosine kinase substrate Eps8, cyclin D1, and CD44 mRNAs, and a substantial up-regulation of phosphatidylinositol 3-kinase p85, IGF-I, and serine protease inhibitor-2.2 mRNAs in SFK cells. The patterns of induction or reduction of gene expression after exposure to bFGF showed considerable overlap between both cell types. Furthermore, both Eps8 and CD44 mRNA levels were reduced greatly in the brain tissues of the cerebrum isolated from the PrP^{-/-} mice. These results indicate that the disruption of the PrP gene resulted in an aberrant regulation of a battery of genes important for cell proliferation, differentiation, and survival, including those located in the Ras and Rac signaling pathways. (*Am J Pathol* 2000, 157:59–68)

Prion diseases are a group of neurodegenerative disorders affecting both animals and humans.¹ The majority of these diseases are transmissible and characterized by

intracerebral accumulation of an abnormal prion protein (PrP^{Sc}) that is identical in the amino acid sequence to the cellular isoform (PrP^C) expressed on the cell surface and attached by a glycosylphosphatidylinositol anchor. The PrP^{Sc} protein differs biochemically from PrP^C by its β -sheet-enriched structure, detergent insolubility, limited proteolysis by proteinase K, and slower turnover rate.¹ It has been proposed that the conversion of PrP^C into PrP^{Sc} is mediated by a homotypic interaction between endogenous PrP^C and incoming or *de novo* generated PrP^{Sc} via an undefined post-translational process.¹ The gene coding for the PrP^C protein is expressed constitutively in a wide variety of neural and nonneural tissues at the highest level in neurons in the central nervous system (CNS), yet little is known about its biological functions.^{2,3} Recent studies indicate that the phylogenetically conserved octarepeat region of the PrP^C protein has a capacity to bind copper, suggesting that PrP^C plays a role in copper metabolism.^{4,5}

Recently, five independent groups have established lines of mice devoid of the PrP^C protein (PrP^{-/-}), designated Zur, Npu, Ngsk, Rcm0, and Rikn, using different gene-targeting strategies.^{6–13} The entire open reading frame of the PrP gene (*Prnp*) was replaced by selectable markers in Ngsk, Rcm0, and Rikn PrP^{-/-} mice, whereas a part of the open reading frame remained intact in Zur and Npu PrP^{-/-} mice. All of these mice exhibited normal early development and complete protection against scrapie infection, indicating that PrP^C, a dispensable protein in embryonic development, is essential for inducing prion diseases.^{6–12} Zur PrP^{-/-} mice showed impairment in the GABA_A receptor-mediated fast inhibition and long-term potentiation in hippocampal CA1 pyramidal neurons, suggesting that PrP^C is necessary for normal synaptic function and plasticity in the hippocampus.^{14,15} However, some investigators were unable to confirm these observations.¹⁶ Both Zur and Npu PrP^{-/-} mice exhibited altered circadian activity rhythms and sleep

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Table 1. Primer Sequences Used for PCR Amplification

Primers	Sequence	Product size (bp)	Genbank accession no.
Eps8 sense	5'TGTCTAACCGCTCCAGTGGGTATG3'	676	L21671
Eps8 antisense	5'CTAACGTCCACCTGTGTGACAGTC3'		
cyclin D1 sense	5'CGTACCCTGACACCAATCTCCTCA3'	641	S78355
cyclin D1 antisense	5'TGTGCGGTAGCAGGAGAGGAAGTT3'		
CD44 sense	5'AATGTAACCTGCCGCTACGCAGGT3'	475	M27129
CD44 antisense	5'AGCCGCTGCTGACATCGTCATCTA3'		
PI3K p85 sense	5'AGTGCAGAGGGCTACCAGTACAGA3'	484	M60651
PI3K p85 antisense	5'GTCGTAATTCTGCAGGGTTGCTGG3'		
IGF-I sense	5'TCGTCTTCACCTCTTCTACCTG3'	369/421	X04480
IGF-I antisense	5'GGTCTTGTTCCTGCACCTCCTCT3'		
Spi-2.2 sense	5'GAAGTCCCAAGTGTGACGCTTC3'	521	M64086
Spi-2.2 antisense	5'GTGGACAAAGTGAGGAGATCCTGC3'		
PrP sense	5'CATTTTGGCAACGACTGGGAGGAC3'	551	M13685
PrP antisense	5'GACTCCATCAAAGGACCTGAAGC3'		
β -actin sense	5'GAGCACAGCTTCTTTGCAGCTCCT3'	255	X03672
β -actin antisense	5'GGTCAGGATACCTCTTGTCTG3'		

PI3K, phosphatidylinositol 3-kinase; IGF-I, insulin-like growth factor-I; Spi, serine protease inhibitor; PrP, prion protein.

patterns.¹⁷ Ngsk, Rcm0, and Rikn PrP^{-/-} mice exhibited late-onset cerebellar ataxia due to an extensive loss of Purkinje cells in the cerebellum.¹⁰⁻¹² Furthermore, Ngsk PrP^{-/-} mice showed a significant amount of demyelination in the spinal cord and peripheral nerves, although the pathophysiological basis for these abnormalities remains unclear.¹⁰⁻¹² The introduction of a wild-type PrP transgene rescued them from Purkinje cell degeneration and demyelination, indicating that PrP^C is directly involved not only in the long-term survival of Purkinje neurons but also in the myelinating capacity of oligodendrocytes and Schwann cells.^{10,11}

The cDNA array technology is a novel approach that allows monitoring of the expression pattern of a large number of genes systematically in a single hybridization, using cDNA probes prepared from different RNA sources on a matrix where a wide variety of cDNA fragments or oligonucleotides are arrayed and immobilized.^{18,19} Previously, using Western blot analysis, we showed that the constitutive and heat-inducible expression of heat shock proteins, HSP105, HSP72, HSC70, HSP60, and HSP25 was similar between two distinct fibroblast cell lines isolated from Ngsk PrP^{-/-} mice and the control PrP^{+/+} mice, suggesting that the PrP^C protein may not act as a cellular regulator during a heat shock response.²⁰ In this study, the gene expression profile was studied in these cell lines by analyzing a cDNA expression array containing 597 clones of various functional classes to elucidate the physiological function of the PrP^C protein.

Materials and Methods

Skin Fibroblast Cell Lines Established from Ngsk Prion Protein-Deficient Mice and Control Mice

The method for producing mice homozygous for a disrupted PrP gene (Ngsk PrP^{-/-} mice) was previously described.⁹⁻¹¹ Three distinct fibroblast cell lines were es-

tablished from abdominal skin explant cultures of the homozygous PrP^{-/-} mice with a mixed 129/Sv × C57BL/6J background (SFK), the heterozygous PrP^{+/-} mice (SFHT), and the control C57BL/6J PrP^{+/+} mice (SFH) at ages 35 to 50 weeks, as described previously.²⁰ Both PrP^{-/-} and PrP^{+/-} mice were produced by intercross between F₁ PrP^{+/-} breeding pairs and their genotypes were determined by Southern blot analysis,⁹⁻¹¹ whereas PrP^{+/+} mice were obtained from Kyudo (Kum-

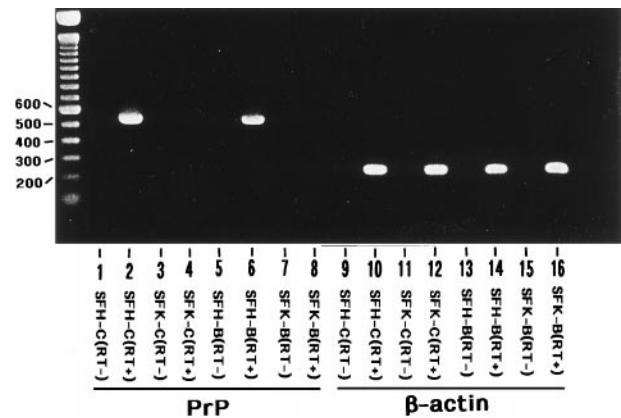


Figure 1. Nonexpression of the PrP gene in a skin fibroblast cell line established from the PrP^{-/-} mice. Two cell lines were established from primary cultures of skin fibroblasts isolated from the PrP^{-/-} mice (SFK) or from the PrP^{+/+} mice (SFH). They were maintained for 4 months *in vitro* before starting the experiments. The cells were incubated for 72 hours in LS medium with (SFH-B and SFK-B) or without (SFH-C and SFK-C) inclusion of 50 ng/ml of bFGF for the last 24 hours before processing for RNA preparation. Fifty nanograms of cDNA prepared from total RNA by reverse transcription (RT) was amplified by PCR using primer pairs specific for the PrP gene (lanes 1–8) or the β -actin gene (lanes 9–16) listed in Table 1. Lanes 1 and 9 represent SFH-C cells processed for RT-PCR omitting the RT step; lanes 2 and 10, SFH-C cells processed for RT-PCR including the RT step; lanes 3 and 11, SFK-C cells processed for RT-PCR omitting the RT step; lanes 4 and 12, SFK-C cells processed for RT-PCR including the RT step; lanes 5 and 13, SFH-B cells processed for RT-PCR omitting the RT step; lanes 6 and 14, SFH-B cells processed for RT-PCR including the RT step; lanes 7 and 15, SFK-B cells processed for RT-PCR omitting the RT step; lanes 8 and 16, SFK-B cells processed for RT-PCR including the RT step. The DNA size marker (bp) is shown on the left.

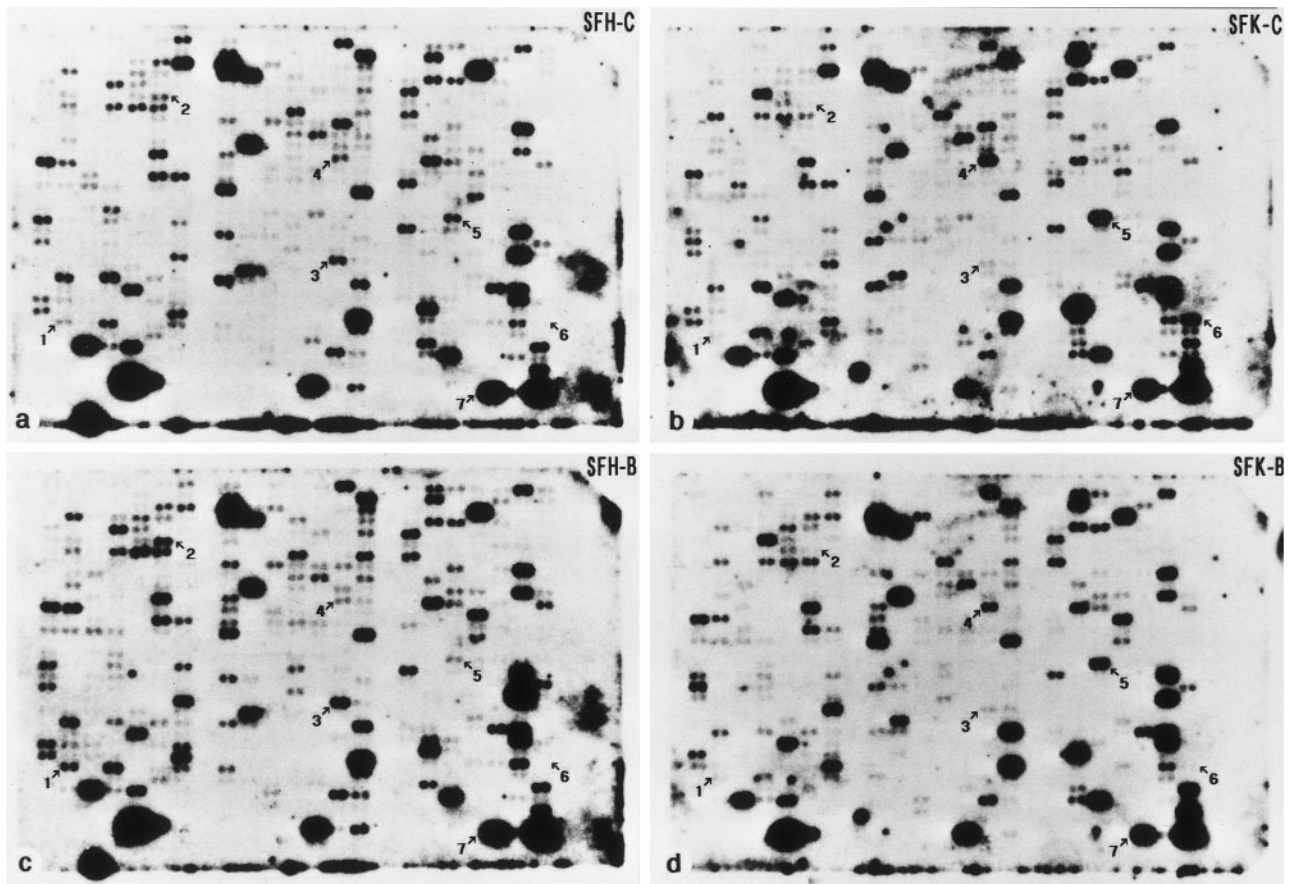


Figure 2. Southern blot hybridization of a cDNA expression array with ³²P-labeled cDNA probes prepared from mRNA isolated from SFK and SFH cells. A set of filters containing 597 arrayed mouse cDNA clones of various functional classes, immobilized as a spot of duplicate dots on a nylon membrane, were hybridized with ³²P-labeled cDNA probes prepared from poly A⁺ RNA that was isolated from bFGF-treated or untreated SFK and SFH cells maintained for 4 months *in vitro*: SFH-C (a), SFK-C (b), SFH-B (c), or SFK-B (d) cells. The spots indicated by arrows represent the clones of (1) the receptor tyrosine kinase substrate Eps8, (2) cyclin D1, (3) CD44, (4) phosphatidylinositol 3-kinase (PI3K) p85, (5) insulin-like growth factor (IGF-1), (6) serine protease inhibitor (Spi-2.2), and (7) β -actin.

amoto, Japan). The cells were plated in 25-cm² culture flasks at a density of 10⁶ cells/flask and incubated at 37°C in a 5% CO₂/95% air incubator in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μ g/ml of streptomycin (feeding medium). The passage of the cultures was performed biweekly. They were maintained for 4 months (SFK, SFH, and SFHT) or 7 months (SFK) before starting the experiments. After the cultures became sub-confluent, the culture medium was replaced by a feeding medium containing 1% FBS instead of 10% FBS; this is the low serum concentration (LS) medium. After a 72-hour incubation in LS medium with or without inclusion of 50 ng/ml of recombinant human basic fibroblast growth factor (bFGF; Sigma, St. Louis, MO) for the final 24 hours, they were processed for RNA preparation.

Extraction of RNA and Synthesis of ³²P-Labeled cDNA Probes

Total RNA was extracted from bFGF-treated SFK, SFH, and SFHT cells (SFK-B, SFH-B, and SFHT-B) and untreated SFK, SFH, and SFHT cells (SFK-C, SFH-C, and SFHT-C), or from the whole cerebral cortices isolated

from the PrP^{-/-} mice (CBRK) and from the PrP^{+/+} mice (CBRH), from which SFK or SFH cells have originated. Poly A⁺ RNA was purified from total RNA pretreated with DNase I, using the oligotex-dT30 latex bead system (Takara, Tokyo, Japan). One microgram of poly A⁺ RNA was reverse-transcribed by incubating at 50°C for 20 minutes in 11 μ l of a reaction mixture containing 50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, 3 mmol/L MgCl₂, 500 μ mol/L dCTP, 500 μ mol/L dGTP, 500 μ mol/L dTTP, 5 μ mol/L dATP, 35 μ Ci of [α -³²P]dATP, 5 mmol/L DTT, 50 units of Moloney murine leukemia virus (MMLV) reverse transcriptase, and the coding sequence primers at a final concentration of 1 \times using a commercial kit (Clontech, Palo Alto, CA) followed by purification through column chromatography.

Southern Blot Hybridization of a cDNA Expression Array

A set of filters containing 597 mouse cDNA clones of various functional classes immobilized as a spot of duplicate dots on a positively charged nylon membrane (Atlas Mouse cDNA Expression Array I, Clontech) were hybridized at 68°C overnight in the hybridization solution

Table 2. Genes Exhibiting a Differential Expression Pattern between SFK and SFH Cells in Analysis of 597 Arrayed cDNA Clones

Genes up-regulated or down-regulated in SFK-C cells compared to SFH-C cells	
Up-regulation (27 clones)	Down-regulation (15 clones)
c-ErbA proto-oncogene	ZO-1 tight junction protein
Cot proto-oncogene	c-myc proto-oncogene
PDGF receptor α	Cyclin B1
Golgi 4-transmembrane spanning transporter	*Cyclin D1
RNA-activating protein kinase inhibitor p58	Thrombin receptor
*Phosphatidylinositol 3-kinase p85	Transferrin receptor protein p90
Glutathione peroxidase	Transcription factor A10
Microsomal glutathione S-transferase	Matrix metalloproteinase-11
Mu 1 glutathione S-transferase	MHR23B UV excision repair protein homologue
Pi 1 glutathione S-transferase	Activating transcription factor 4
Serine proteinase inhibitor SPI3	*Eps8 receptor tyrosine kinase substrate
Adipocyte differentiation-associated protein	*CD44
p45 NF-E2-related transcription factor	Vascular cell adhesion molecule-1
Transcription factor S-II	IGF-binding protein-4
Monocyte chemoattractant protein-3	Ornithine decarboxylase
Growth hormone receptor	
Integrin β 1	
IGF-binding protein-3	
IGF-binding protein-5	
IGF-binding protein-6	
*IGF-I	
TGF- β 2	
Cathepsin B	
Cathepsin D	
*Serine protease inhibitor-2.2	
Serine protease inhibitor-2.4	
Tissue inhibitor of metalloproteinase-3	

Genes Up-regulated or Down-regulated in SFH-B Cells Compared to SFH-C Cells	
Up-regulation (25 clones)	Down-regulation (15 clones)
c-myc proto-oncogene	Cot proto-oncogene
Net transcription factor	Cdk4/cdk6 inhibitor p18
Lfc proto-oncogene	Prothymosin α
Cyclin B2	Protein kinase C- θ
*Cyclin D1	*Phosphatidylinositol 3-kinase p85
Etoposide-induced p53 responsive mRNA	Glutathione reductase
14-3-3 η	Homeobox protein-2.1
Microsomal glutathione S-transferase	Homeobox protein-8
Gadd45	C5A receptor
Serine proteinase inhibitor SPI3	Cannabinoid receptor 2
PA6 stromal protein	P-selectin
MHR23B UV excision repair protein homologue	IGF-binding protein-1
*Eps8 receptor tyrosine kinase substrate	IGF-binding protein-5
SRY-box containing gene 4	*IGF-I
Transcription factor SEF2	FGF-7
Transcriptional enhancer factor-1	
YB1 DNA binding protein	
Monocyte chemoattractant protein-3	
Insulin receptor substrate-1	
LDL receptor	
*CD44	
Integrin β 1	
Vascular cell adhesion molecule-1	
Vimentin	
Cathepsin L	

(Table continues)

containing the ^{32}P -labeled cDNA probes at a concentration of 1×10^6 cpm/ml, according to the methods described previously.²¹ The membranes were exposed to Kodak BioMax MS X-ray films with the intensifying screen at -80°C for 24 hours. For rehybridization, the probes were stripped from the membranes by washing in boiled 0.5% sodium dodecyl sulfate solution for 15 minutes. Densitometric analysis was performed on an imaging

system using NIH Image version 1.61 software. The signal intensities were standardized against those of a housekeeping gene, β -actin.

RT-PCR Analysis and Northern Blot Analysis

For reverse transcription-polymerase chain reaction (RT-PCR) analysis, 5 μg of total RNA pretreated with DNase I

Table 2. Continued

Genes Up-regulated or Down-regulated in SFK-B Cells Compared to SFK-C Cells	
Up-regulation (17 clones)	Down-regulation (22 clones)
c- <i>myc</i> proto-oncogene	c-ErbA proto-oncogene
Lfc proto-oncogene	Cot proto-oncogene
Cyclin B1	Cdk4/cdk6 inhibitor p18
Cyclin B2	Prothymosin α
Etoposide-induced p53-responsive mRNA	Protein kinase C- θ
Glucose regulated protein 78	*Phosphatidylinositol 3-kinase p85
gp130	Glutathione peroxidase
MAP kinase kinase-1	Glutathione reductase
14-3-3 η	AT motif-binding factor 1
Serine proteinase inhibitor SPI3	BKLF CACCC box-binding protein
Matrix metalloproteinase-11	Homeobox protein-2.1
MHR23B UV excision repair protein homologue	Homeobox protein-8
Adipocyte differentiation-associated protein	Bone morphogenic protein receptor
Transcription factor SEF2	C5A receptor
Integrin β 1	Cannabinoid receptor 2
Laminin receptor 1	P-selectin
Serine protease inhibitor homologue J6	IGF-binding protein-3
	*IGF-I
	TGF- β 2
	Cytotoxic T lymphocyte-specific serine protease CTLA-1
	*Serine protease inhibitor-2.2
	Serine protease inhibitor-2.4

*The mRNA levels of Eps8, cyclin D1, CD44, phosphatidylinositol 3-kinase (PI3K) p85, IGF-I, and serine protease inhibitor (Spi-2.2) in SFK and SFH cells were also analyzed by Northern blotting as shown in Figure 3A. Both SFK and SFH cells were maintained for 4 months *in vitro* before starting the experiments.

was processed for cDNA synthesis using oligo(dT)₁₂₋₁₈ primers and SuperScript II reverse transcriptase (Gibco BRL, Gaithersburg, MD). Fifty nanograms of cDNA were amplified by PCR using the specific sense and antisense primers listed in Table 1. For Northern blot analysis, 2 μ g (SFK, SFH, and SFHT) or 6 μ g (CBRK and CBRH) of total RNA was separated on a 1.5% agarose-6% formaldehyde gel, transferred onto nylon membranes, and immobilized by UV fixation as described previously.^{21,22} The membranes were hybridized at 53°C overnight in the hybridization buffer containing a digoxigenin (DIG)-labeled DNA probe synthesized using a PCR DIG probe synthesis kit (Boehringer Mannheim, Mannheim, Germany) and the specific sense and antisense primers listed in Table 1. The membranes were further processed for rehybridization with the DIG-labeled DNA probe specific for the β -actin gene to normalize the reaction. The specific reaction was visualized using the DIG chemiluminescence detection kit (Boehringer Mannheim).

Results

Nonexpression of the PrP Gene in SFK Cells Derived from the PrP^{-/-} Mice

With RT-PCR analysis, the expression of PrP mRNA was undetectable in bFGF-treated and untreated SFK cells (SFK-B and SFK-C), whereas it was identified in SFH cells derived from the PrP^{+/+} mice under both culture conditions, SFH-B and SFH-C (Figure 1, lanes 2, 4, 6, and 8). In contrast, the expression of β -actin mRNA was observed in both SFK and SFH cells (Figure 1, lanes 10, 12,

14, and 16). No products were amplified in total RNA samples processed for PCR when the reverse transcription step was omitted, confirming that a contamination of genomic DNA was excluded (Figure 1, lanes 1, 3, 5, 7, 9, 11, 13, and 15).

Identification of Differentially Expressed Genes between SFK and SFH Cells by Analyzing a cDNA Expression Array

The cDNA expression array was analyzed by Southern blot hybridization with the ³²P-labeled cDNA probes prepared from poly A⁺ RNA which was isolated from SFH-C, SFK-C, SFH-B or SFK-B cells, all of which were maintained for 4 months *in vitro*. Among the 597 cDNA clones, 42 genes exhibited a differential expression pattern between SFK-C and SFH-C cells (Figure 2, a and b, and Table 2). The expression of 15 genes including those essential for cell proliferation and adhesion, such as c-*myc* proto-oncogene, cyclin D1, the receptor tyrosine kinase substrate Eps8, CD44, and vascular cell adhesion molecule-1, was reduced in SFK-C cells compared to the levels in SFH-C cells (Table 2). In contrast, the expression of 27 genes including those involved in the insulin-like growth factor-I (IGF-I) signaling pathway, such as phosphatidylinositol 3-kinase (PI3K) p85, IGF-binding proteins (IGFBP) -3, -5, and -6, and IGF-I, was elevated in SFK-C cells compared to the levels in SFH-C cells (Table 2). Exposure to bFGF elevated the expression levels of 25 genes in SFH cells and 17 genes in SFK cells by up-regulating eight genes shared between both, such as

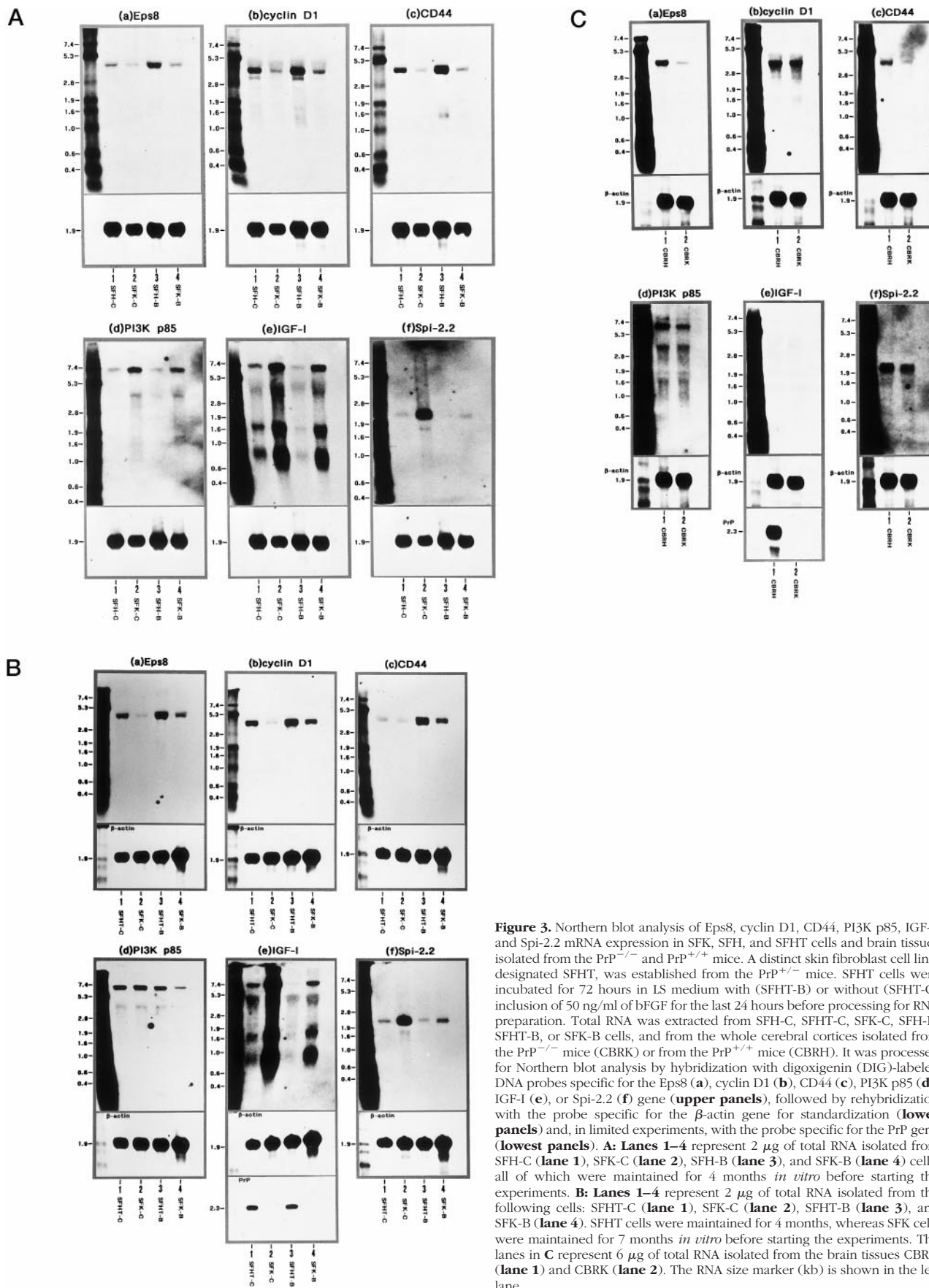


Figure 3. Northern blot analysis of Eps8, cyclin D1, CD44, PI3K p85, IGF-I, and Spi-2.2 mRNA expression in SFK, SFH, and SFHT cells and brain tissues isolated from the PrP^{-/-} and PrP^{+/+} mice. A distinct skin fibroblast cell line, designated SFHT, was established from the PrP^{+/+} mice. SFHT cells were incubated for 72 hours in LS medium with (SFHT-B) or without (SFHT-C) inclusion of 50 ng/ml of bFGF for the last 24 hours before processing for RNA preparation. Total RNA was extracted from SFH-C, SFHT-C, SFK-C, SFH-B, SFHT-B, or SFK-B cells, and from the whole cerebral cortices isolated from the PrP^{-/-} mice (CBRK) or from the PrP^{+/+} mice (CBRH). It was processed for Northern blot analysis by hybridization with digoxigenin (DIG)-labeled DNA probes specific for the Eps8 (a), cyclin D1 (b), CD44 (c), PI3K p85 (d), IGF-I (e), or Spi-2.2 (f) gene (upper panels), followed by rehybridization with the probe specific for the β -actin gene for standardization (lower panels) and, in limited experiments, with the probe specific for the PrP gene (lowest panels). **A:** Lanes 1–4 represent 2 μ g of total RNA isolated from SFH-C (lane 1), SFK-C (lane 2), SFH-B (lane 3), and SFK-B (lane 4) cells, all of which were maintained for 4 months *in vitro* before starting the experiments. **B:** Lanes 1–4 represent 2 μ g of total RNA isolated from the following cells: SFHT-C (lane 1), SFK-C (lane 2), SFHT-B (lane 3), and SFK-B (lane 4). SFHT cells were maintained for 4 months, whereas SFK cells were maintained for 7 months *in vitro* before starting the experiments. The lanes in C represent 6 μ g of total RNA isolated from the brain tissues CBRH (lane 1) and CBRK (lane 2). The RNA size marker (kb) is shown in the left lane.

14-3-3 η and the UV excision repair protein homologue MHR23B (Figure 2, a-d, and Table 2). Treatment with bFGF reduced the levels of expression of 15 genes in SFH cells and 22 genes in SFK cells by down-regulating 12 genes shared between both, such as cdk4/cdk6 inhibitor p18 and protein kinase C- θ (Figure 2, a-d, and Table 2).

Northern Blot Analysis of Six Genes Expressed Differentially between SFK and SFH Cells

Since it is impractical to quantify all 42 genes expressed differentially between SFK and SFH cells under untreated conditions using Northern blot analysis, six clones exhibiting a great difference between both were selected in view of their potential involvement in cell proliferation, differentiation, and survival. They included three genes down-regulated in SFK-C cells *versus* SFH-C cells: Eps8 (Figure 2, spot 1), cyclin D1 (Figure 2, spot 2), and CD44 (Figure 2, spot 3) and three genes up-regulated in SFK-C cells *versus* SFH-C cells: PI3K p85 (Figure 2, spot 4), IGF-I (Figure 2, spot 5), and the serine protease inhibitor (Spi)-2.2, a mouse equivalent to the human α 1-antichymotrypsin (ACT)²³ (Figure 2, spot 6). In Northern blotting, the levels of expression of Eps8 (4.7 kb), cyclin D1 (4.5 and 3.8 kb), and CD44 (4.7 kb) mRNAs were reduced in SFK-C cells to 33%, 28%, or 22% of those in SFH-C cells, respectively, when standardized against corresponding β -actin signals detected on the identical blots (Figure 3A, a-c, lanes 1 and 2). After exposure to bFGF, the levels of Eps8, cyclin D1, and CD44 mRNAs were elevated in SFH-B cells by 3.2-fold, 1.4-fold, or 1.9-fold, respectively, compared to those in SFH-C cells, and increased in SFK-B cells by 2.4-fold, 1.3-fold, or 2.1-fold, respectively, compared to those in SFK-C cells (Figure 3A, a-c, lanes 1-4). However, the levels of Eps8, cyclin D1, and CD44 mRNAs were much lower in SFK-B cells than those in SFH-C cells (Figure 3A, a-c, lanes 1-4). In contrast, the levels of PI3K p85 (7.0 and 4.0 kb), IGF-I (7.0, 1.6, and 0.8 kb) and Spi-2.2 (2.1 kb) mRNAs were elevated in SFK-C cells by 4.6-fold, 6.0-fold, or 25.7-fold, respectively, compared to those in SFH-C cells (Figure 3A, d-f, lanes 1 and 2). After bFGF exposure, the expression of PI3K p85, IGF-I and Spi-2.2 mRNAs was reduced in SFK-B cells to 59%, 85%, or 6% of those in SFK-C cells, and decreased in SFH-B cells to 71%, 17%, or 16% of those in SFH-C cells, respectively, with the greatest reduction of Spi-2.2 expression in SFK cells after bFGF treatment (Figure 3A, d-f, lanes 1-4).

Effects of Culture Periods and Strain Differences on Gene Expression Levels in Skin Fibroblast Cell Lines

To examine the possible effects of strain differences (129/Sv \times C57BL/6J background in SFK *versus* C57BL/6J background in SFH) on the gene expression levels, a distinct cell line designated SFHT was established from the skin explant cultures of the heterozygous

PrP^{+/-} mice that were produced by intercross between F₁ PrP^{+/-} breeding pairs.⁹⁻¹¹ The expression of Eps8, cyclin D1, CD44, PI3K p85, IGF-I, and Spi-2.2 mRNAs was studied between bFGF-treated or untreated SFHT cells (SFHT-B and SFHT-C) maintained for 4 months *in vitro* and SFK cells (SFK-B and SFK-C) maintained for 7 months *in vitro* (Figure 3B, a-f, lanes 1-4). In Northern blotting, the patterns of expression of these mRNAs were almost identical between SFK cells maintained for 4 months and those maintained for 7 months *in vitro* (Figure 3A, a-f, and 3B, a-f, lanes 2 and 4), indicating that the culture periods might not constitute a major factor contributing to the aberrant gene expression identified in SFK cells. Furthermore, there was a high degree of similarity between the patterns of expression of these mRNAs in SFHT-C and SFK-C cells and those in SFH-C and SFK-C cells examined in the initial experiments, except that SFHT-C cells expressed a lower level of CD44 mRNA and a higher level of PI3K p85 mRNA than SFH-C cells (Figure 3A, a-f, and 3B, a-f, lanes 1 and 2). The levels of expression of Eps8, cyclin D1, and CD44 mRNAs in SFK-C cells maintained for 7 months were reduced to 6%, 9%, or 34% of those in SFHT-C cells, respectively (Figure 3B, a-c, lanes 1 and 2). In contrast, the levels of IGF-I and Spi-2.2 mRNAs were elevated in SFK-C cells by 4.9-fold or 4.2-fold, respectively, compared to those in SFHT-C cells (Figure 3B, e and f, lanes 1 and 2). PrP mRNA was undetectable in SFK cells but detectable in SFHT cells (Figure 3Be, lanes 1-4). These results suggest that the aberrant gene expression identified in SFK cells is unlikely to be due to the effects of strain differences.

Differential Gene Expression in Brain Tissues of the PrP^{-/-} Mice and PrP^{+/+} Mice

To evaluate the possibility that the differential gene expression between SFK and SFH cells might represent a fibroblast cell line-specific observation, the expression of Eps8, cyclin D1, CD44, PI3K p85, IGF-I, and Spi-2.2 mRNAs was studied in the brain tissues of the cerebrum isolated from the PrP^{-/-} mice (CBRK) and the PrP^{+/+} mice (CBRH) from which SFK or SFH cells originated. In Northern blotting, the levels of expression of Eps8 and CD44 mRNAs in CBRK were reduced to 20% or 18% of those in CBRH, when standardized against corresponding β -actin signals detected on the identical blots (Figure 3C, a and c, lanes 1 and 2). Both PI3K p85 and Spi-2.2 mRNA levels in CBRK were decreased slightly to 83% or 88% of those in CBRH, whereas cyclin D1 mRNA expression was not significantly different between both (Figure 3C, b, d, and f, lanes 1 and 2). IGF-I mRNA was identified in neither of them, whereas PrP mRNA was detectable in CBRH but undetectable in CBRK (Figure 3Ce, lanes 1 and 2).

Discussion

By analyzing a cDNA expression array containing 597 clones, this study showed that a number of biologically

important genes exhibited a differential expression pattern between two fibroblast cell lines: SFK derived from the PrP^{-/-} mice and SFH derived from the PrP^{+/+} mice. When SFK cells were compared with SFH cells without bFGF treatment, the expression of 15 genes, including those essential for cell proliferation and adhesion, was reduced, whereas the expression of 27 genes, including those involved in the IGF-I signaling pathway, was elevated. The patterns of induction or reduction of gene expression after exposure to bFGF showed a considerable overlap between both. Among the differentially expressed genes, Northern blot analysis verified a significant down-regulation of Eps8, cyclin D1, and CD44 mRNAs and a substantial up-regulation of PI3K p85, IGF-I and Spi-2.2 mRNAs in SFK cells, supporting the validity of the cDNA expression array analysis for screening the differential gene expression. The observations on SFHT cells established from the PrP^{+/-} mice and SFK cells maintained for a longer period in culture indicated that the aberrant gene expression identified in SFK cells is unlikely to be due to the effects of culture periods and strain differences, but is most likely to be attributable to the homozygous disruption of the PrP gene in these cells. Furthermore, both Eps8 and CD44 mRNA levels were reduced greatly in the cerebral tissues isolated from the PrP^{-/-} mice compared to those of the PrP^{+/+} mice. These results suggest that the PrP^C protein plays an important role in regulating gene expression involved in cell proliferation, differentiation and survival, both *in vitro* and *in vivo*, because these genes encode key molecules located within or very close to the Ras and Rac signaling cascades.

The Ras proteins, activated after cell exposure to growth factors, integrate a number of downstream effectors, leading to an activation of the Raf/MAP kinase pathway, the Ral-GDS pathway, and the PI3K pathway.²⁴ Eps8 is originally identified as a widely expressed substrate for the receptor tyrosine kinases (RTKs).²⁵ Its overexpression in cultured fibroblasts enhances mitogenic responsiveness to endothelial growth factor.²⁵ Eps8 mRNA and protein expression is induced markedly in cultured fibroblasts by exposure to serum or phorbol esters, whereas it is suppressed in C2C12 myoblast cells after terminal differentiation.²⁶ Eps8 has a Src homology region 3 (SH3) domain that functions as an adapter, assembling intracellular signal-transducing molecules with RTKs.²⁵ Eps8 plays a pivotal role in signal transduction from both Ras and PI3K to Rac, a member of the subfamily of Rho-GTPases.²⁷ The Rac proteins that constitute the NADPH oxidase complex and act as a regulator of actin cytoskeletal reorganization have a role in cell cycle progression by activating the cyclin D1 promoter after the generation of intracellular reactive oxygen species through NF- κ B activation.²⁸ Cyclin D1 expression is up-regulated in MCF7 human breast cancer cells by exposure to IGF-I, followed by hyperphosphorylation of a retinoblastoma protein Rb, whereas it is suppressed by PI3K-specific inhibitors.²⁹ CD44, a cell surface glycoprotein expressed in both hematopoietic and nonhematopoietic cell types, is involved in cell adhesion by interacting with the extracellular matrix glycosaminoglycans fi-

bronectin, laminin, and collagen. The expression of CD44 protein or its alternatively spliced variants is elevated in certain tumor cells with metastatic potential and in Ras-transformed fibroblasts in culture.³⁰ All of these observations could support the hypothesis that the down-regulation of Eps8, cyclin D1, and CD44 in SFK cells might reflect a functional impairment in the Ras and Rac signaling pathways in these cells.

The PI3K protein is composed of a 110-kd catalytic subunit (p110) and an 85-kd regulatory subunit (p85), the latter containing one SH3 domain and two SH2 domains by which it links to multiple signaling components. The PI3K protein, located immediately downstream from Ras, plays a role in insulin-stimulated glucose transport, exocytosis, neurite outgrowth, prevention of apoptosis, and cell cycle progression.²⁴ The downstream targets of PI3K are the Rac proteins, the ribosomal protein kinase p70^{S6K}, and the serine/threonine protein kinase PKB/Akt. IGF-I activates PI3K, followed by activation of PKB/Akt, which promotes IGF-I-dependent survival of cerebellar neurons in culture.³¹ Spi-2.2, a mouse homologue of the human ACT, belongs to a multigene family composed of closely related Spi-encoding genes derived by ancestral gene duplication. The expression of both Spi-2.1 and Spi-2.3 genes are up-regulated by growth hormone (GH) or by IGF-I, whereas Spi-2.2 gene expression is not GH-dependent but is induced after acute inflammatory reactions through activation of a panel of signal transducers and activators of transcription (STAT) proteins.^{23,32} IGF-I stimulates the release of ACT in MCF7 cells.³³ All of these observations put forth a possible scenario that the up-regulated expression of PI3K p85, IGF-I, and Spi-2.2 mRNAs in SFK cells might represent a compensatory response to the reduced levels of cyclin D1 to maintain the cellular biological functions mediated by the IGF-I signaling pathway.³⁴ The regulatory mechanisms underlying IGF-I gene expression are highly complex, and include the use of alternative promoters acting on multiple initiation sites, differential RNA splicing, polyadenylation, and translation.³⁵ The biological activities of IGF-I are regulated by its half-life and affinity for specific receptors, which are affected by IGF-BPs.³⁵ IGF-BPs are cleaved by a family of serine proteases into non-IGF-I-binding fragments.³⁶ It is worth noting that the expression of a panel of IGF-BP mRNAs is found to be elevated in SFK cells.

The mechanisms by which an absence of the PrP^C protein in SFK cells deregulates the expression of a battery of genes located in the Ras and Rac signaling pathways remain unknown. The PrP^C protein is enriched in a specialized compartment consisting of detergent-insoluble, cholesterol-rich membranous microdomains called caveolae-like domains (CLDs) or rafts, where a battery of signaling molecules including platelet-derived growth factor and endothelial growth factor receptors, Ras, PI3K, and nitric oxide synthase are clustered.^{37,38} PrP^C interacts with PrP^{Sc} in this compartment during the formation of nascent PrP^{Sc} protein.^{37,38} PrP-deficient cells in culture showed an inability to deal with oxidative stress due to reduced activity of Cu/Zn superoxide dismutase, suggesting that the function of PrP^C is important for cellular

resistance to oxidative stress.^{4,39} A recent study using hippocampal neuronal progenitor cell lines isolated from Rikn PrP^{-/-} mice has revealed that serum removal from these cultures facilitates apoptosis.¹³ Another study has shown that neuronal nitric oxide synthase is lost from the rafts in cerebellar tissues of adult Zur PrP^{-/-} mice.⁴⁰ All of these observations suggest that PrP^C might play a role in the organization of signaling complexes in CLDs (rafts), and the deficiency of its function might disturb the CLD- (raft-) associated signal transduction that is pivotal for protection against oxidative stress and apoptosis, or for synaptic transmission, although further studies are required to evaluate this hypothesis.

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