

RESEARCH ARTICLE

2-DE proteome analysis of a proliferating and differentiating human neuronal stem cell line (ReNcell VM)

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The proteome of a proliferating human stem cell line was analyzed and then utilized to detect stem cell differentiation-associated changes in the protein profile. The analysis was conducted with a stable human fetal midbrain stem cell line (ReNcell VM) that displays the properties of a neural stem cell. Therefore, acquisition of proteomic data should be representative of cultured human neural stem cells (hNSCs) in general. Here we present a 2-DE protein-map of this cell line with annotations of 402 spots representing 318 unique proteins identified by MS. The subsequent proteome profiling of differentiating cells of this stem cell line at days 0, 4 and 7 of differentiation revealed changes in the expression of 49 identified spots that could be annotated to 45 distinct proteins. This differentiation-associated expression pattern was validated by Western blot analysis for transgelin-2, proliferating cell nuclear antigen, as well as peroxiredoxin 1 and 4. The group of regulated proteins also included NudC, ubiquilin-1, STRAP, stress-70 protein, creatine kinase B, glial fibrillary acidic protein and vimentin. Our results reflect the large rearrangement of the proteome during the differentiation process of the stem cells to terminally differentiated neurons and offer the possibility for further characterization of specific targets driving the stem cell differentiation.

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Abbreviations: EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; hNSC, human neural stem cells; NSC, neural stem cells; PCNA, proliferating cell nuclear antigen

1 Introduction

Stem cells are defined by several unique properties: they are unspecialized cells, capable of dividing and renewing themselves for long periods of time (self-renewal), and they can give rise to many cell types such as blood, nerve, and muscle cells. Whereas embryonic stem cells, which are derived from very early embryos (blastocysts), are totipotent, *i.e.* they are

capable of generating all cell types of the body, adult stem cells have a restricted potential to differentiate into certain cell types. The latter are present at all stages of development and probably exist in all multicellular organisms.

Replacement of lost neurons by cell transplantation of neural stem cells (NSC) constitutes a promising new approach to the treatment of progressive neurodegenerative diseases, like Parkinson's disease, Huntington's disease, stroke, Alzheimer's disease and probably multiple sclerosis. However, it becomes increasingly difficult to draw consistent conclusions from published and ongoing studies mainly because of the differences in the cell types and the preparation protocols applied. For example, rodent neural stem cells are distinctly different in their properties from the human NSC and the data generated are not necessarily comparable [1, 2]. The variable results obtained from human fetal-derived NSC can probably be attributed to the different age, quality and preparation method of the NSC derived from different tissue samples.

However, *in vitro* generation of stable, renewable and consistently functional specific NSC lines will prove to be particularly beneficial in the studies required to move towards cell therapy, and various immortalized neural stem cell lines have been established [3–5]. Furthermore, the *in vitro* storage, expansion and characterization of such NSC are very likely to alleviate the ethical and logistic problems associated with the use of fresh embryonic tissue. Using a number of variations in culture conditions and/or genetic modifications, these cultured cells can be differentiated into different neuronal progeny.

The commitment of stem cells to differentiate as well as the maturation involves dramatically complex events leading to the generation of different phenotypes via distinctive developmental programs. These regulatory changes can be studied systematically with transgenic technology and microarray techniques [6–9]. However, ultimately, the regulatory functions are carried out by the cellular protein constituents, and thus comprehensive proteome expression profiling will likely provide important clues to the understanding of the differentiation processes [10–12]. Proteomic profiling has previously mainly been applied for the study of the protein inventory in the adult nervous system in health and disease [13–19]. Even if transcriptional profiling currently provides a more comprehensive coverage, only proteomic approaches will address such important issues such as protein amount, protein stability, sub-cellular localization of proteins, post-translational modifications and protein–protein interactions.

Maurer and coworkers [20] presented a proteomic database for NSC isolated from the brains of adult rats and cultured for 10 weeks. The authors were able to map about 1100 protein spots in a 2-DE proteome profiling approach of which 266 were identified.

However, up to now human fetal neural stem cells have not been subjected to profound proteome analysis, although they have a great therapeutic capacity [21–23]. Furthermore,

the proteome map of neural stem cells will prove to be a valuable tool for the investigation of signal modulation for directed differentiation and will provide quality control criteria for immortalized cell lines. Applying proteome approaches, the programs that control self-renewal, differentiation, and plasticity will be accessible at a comprehensive scale. In addition, new specific markers for neuronal development and factors driving the differentiation of stem cells in defined directions may be identified.

However, although human neural stem cells (hNSC) are available for research purposes, stable neural stem cell lines have not been extensively characterized. To meet this need, using the ReNcell VM model we now want to provide a proteome reference database for human neural midbrain stem cells that constitutes the basis for further investigations of differential protein expression during differentiation. This neural stem cell line is nestin-positive in the undifferentiated state and displays neural lineage restricted multi-potentiality upon differentiation (manuscript in preparation, ReNeuron Limited, Guildford, Surrey, UK). ReNcell VM can be cultured as proliferating undifferentiated cells and reliably differentiated into neurons, astrocytes and oligodendrocytes *in vitro*. Thus, proteome information of this cell line is of general use for the understanding of stem cells and might ultimately lead to the design and selection of future cell lines for the treatment of neurodegenerative diseases such as Parkinson's disease.

2 Materials and methods

2.1 Immortalized ventral mesencephalic cell line (ReNcell VM)

ReNcell VM is an immortalized human neural stem cell line (ReNeuron Ltd., Guildford, Surrey, UK) derived from the ventral mesencephalon from 10-week fetal neural tissue. The cell line was established following immortalization with the v-myc oncogene by retroviral transduction. Non-genetically modified human NSC *in vitro* cultures have reduced telomerase activity leading to genetic instabilities [24]. Transformation using the myc transcription factor has proven highly effective at extending the normal lifespan of human NSC *in vitro* and maintaining a stable genotype and phenotype [19]. ReNcell VM cells were infected with high titer amphotropic retrovirus (TEFLY-A) carrying the immortalizing transgene v-myc plus a selection marker and were selected for 2 weeks with 150 µg/mL G418 (Invitrogen, Paisley, UK). The cells are readily expandable with a doubling time of about 24 h. They reveal chromosomal stability over >200 population doublings. The cell line was selected after *in vitro* assays for stable karyotype, proliferation (CyQuant, Molecular Probes) and neural differentiation with Western blot and immunofluorescence detection of nestin for immature neuronal progenitors, glial fibrillary acidic protein (GFAP) for astrocytic glial cells, β -III tubulin for mature neuronal cells,

tyrosine hydroxylase (TH) for catecholaminergic neurons and Hoechst 33342 for nuclear DNA, as well as robustness (freeze-thaw viability). The differentiation resulted in about 50% glial (mainly astrocytic) cells and 50% neurons, 10% of which had a dopaminergic phenotype (manuscript in preparation, ReNeuron).

2.2 Cell growth and differentiation

A standard differentiation protocol was used to differentiate the stem cell monolayer of paving stone morphology into a differentiated cellular neural/glial network. Briefly, after thawing and resuspension of the ReNcell VM cells with media [DMEM:F12 with B27 supplement mix, L-glutamine, gentamycin (all from Invitrogen, Karlsruhe, Germany) and heparin (Sigma, Munich, Germany)] the cell viability was analyzed. Subsequently, cells were spun down, resuspended again in fresh media, and plated in a dish. A 4-day cultivation (37°C) with growth factors [epidermal growth factor (EGF), basic fibroblast growth factor; Peprotech, London, UK] was performed to reach confluency on laminin-coated (1 to 5 µg/cm²) plastic surface.

To passage the cells each culture was rinsed once with calcium- and magnesium-free HBSS solution, and then 2.5 mL of trypsin solution (BioWhittaker, USA) was added, and the cultures were incubated for 20 min at 37°C. As soon as the cells rounded up, the culture dishes were gently tapped to promote detachment of cells and trypsin was inactivated with the addition of culture medium. The cell suspensions were transferred to fresh tubes, rinsed again with media and centrifuged at 500 × g for 5 min at 4°C in a swinging bucket bench-top centrifuge. After removal of the supernatant, the pellets were resuspended five times in 1 mL of media. A cell count and viability determination was performed using a hemocytometer and cells diluted 1:1 in 0.4% trypan blue. Finally, cell suspensions were diluted in media (containing basic fibroblast growth factor 1 µL/mL; EGF 2 µL/mL) to an appropriate cell concentration (250 000 to 500 000 cells per 10-cm dish), plated in laminin-coated tissue culture plates and returned into the incubator at 37°C. Differentiation of the cells was achieved by growing the cells in the presence of mitogens for 3–4 days followed by the withdrawal of growth factors in culture medium. Within 2–3 days of removal of the growth factors, the cells begin to change to a more differentiated morphology. Cells were harvested for proteomics analysis undifferentiated and after 4 and 7 days of differentiation, respectively.

2.3 Protein preparation

For the preparation of crude protein extracts culture medium was removed from the ReNcell VM cells and the adherent cells were washed once with 5 mL PBS followed by an additional short rinsing with ice-cold double distilled H₂O to remove remaining proteins. The complete tissue culture dish was quickly frozen with liquid nitrogen, allowed to

warm up to room temperature and 1 mL lysis buffer containing 7 M urea, 2 M thiourea, 70 mM DTT, 4% w/v CHAPS and 1.5 mg/mL Complete[®] and PMSF protease inhibitor (all reagents from Roche, Mannheim, Germany) were added. The suspension was transferred to fresh tubes, quickly frozen with liquid nitrogen and sonicated in an ice-cold ultrasonic bath for 30 min to achieve optimum protein extraction. Subsequently, the lysate was centrifuged at 13 000 rpm for 5 min at 4°C. Protein content of the supernatant was measured with an Ettan 2-D Quant Kit (Amersham Biosciences, Uppsala, Sweden). Sample aliquots were stored at –80°C.

2.4 2-DE

Aliquots of the protein samples, 150 µg for silver-stained gels and 500 µg for colloidal Coomassie-stained gels, were precipitated with chloroform/methanol and resuspended in 420 µL rehydration buffer containing 8 M urea, 2% CHAPS, 1.3% IPG-buffer and 16.2 mM DTT. The samples were passively rehydrated overnight followed by the IEF procedure on 24-cm non-linear pH 3–10 immobilized gradient strips (Immobiline DryStrips, Amersham Biosciences) by using the Protean IEF Cell (Bio-Rad, Hercules, CA, USA). Electro focusing was carried out at 8000 V resulting in approximately 95 kVh in total. After IEF, proteins were reduced for 20 min at room temperature using 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 10 mg/mL DTT followed by alkylation for 20 min with the same buffer containing 40 mg/mL iodacetamide instead of DTT. The second dimension was carried out on 12.5% SDS PAGE gels (25 cm × 22.5 cm × 0.5 cm) and 8 W/gel at 15°C. Gels were fixed with 10% acetic acid, 50% ethanol/40% double distilled H₂O and 0.1 mL/L of 37% formaldehyde for silver gels. Analytical gels were stained using silver nitrate according to Blum *et al.* [25] and preparative gels for mass spectrometric analysis were stained with colloidal CBB G-250 (Serva, Heidelberg, Germany). Silver- and CBB-stained gel images were digitized using Microtec ScanMaker 9600 XL.

2.5 Spot detection and quantitation

Gel evaluations were performed with the Delta2D software version 3.2 (Decodon, Greifswald, Germany). For identification of the differentially expressed protein, spots of two replicate silver-stained gels of each of the time points were first matched to each other and merged to fused images. In a second step, these fused images of the different time points (0, 4 and 7 days of differentiation) were fused again, resulting in a merged image from all time points. By choosing the “average” option for the fusion process, the relative intensities of the spots were kept in the fused image. A spot detection was performed with the Delta 2D software for the fused image of all time points [26]. Spots of the fused gel were manually edited by comparing the original gel images with the fused image and subsequently the spot map and the

corresponding labels were propagated to each of the three fused gel images (0, 4 and 7 days of differentiation) included in the analysis.

Spot intensities were determined and normalized. The relative intensity of each spot was calculated by dividing the intensity of each spot by the sum of the intensities of all spots on the corresponding gel. Induction / repression ratios between the different time points were calculated by dividing these relative intensities. For spot isolation and subsequent protein identification from the CBB-stained gels, the spot pattern of gels stained with silver nitrate or colloidal CBB were matched, thus assuring unequivocal assignment of spots. Thereby each cell culture gave rise to one CBB gel and two analytical silver-stained gels.

2.6 Preparation of tryptic peptide mixtures

Protein spots were excised from colloidal CBB-stained 2-D gels using spot pickers (Flexys Proteomics picker, Genomic Solutions, Ann Arbor, MI, USA or Proteome Works™, Bio-Rad, respectively) and transferred to 96-well plates. For the MALDI-MS measurements with a Reflex III mass spectrometer (Bruker Daltonics, Bremen, Germany) digestion with trypsin was performed manually, using specific 96-well plates (Genomic Solutions) and a low-salt procedure adapted from the methods of Fountoulakis and Langen [27] and Nordhoff and coworkers [28]. Manually digested peptide samples were spotted for MALDI-MS measurement onto Bruker AnchorChip™ (384/600 μm) according to the method being published from Nordhoff and coworkers in 2003 [29]. For MALDI-MS/MS measurements with a 4700 Proteomics-Analyzer (Applied Biosystems, Foster City, CA, USA) the digestion and spotting of the peptide mixtures to the MALDI target were done automatically with the Ettan Spot Handling Workstation (Amersham-Biosciences). For both MS-methods CHCA was used as matrix solution.

2.7 MALDI-TOF-MS analysis

Peptide mixtures were analyzed by MALDI-TOF-MS using a Reflex III mass spectrometer (Bruker Daltonics) operating in positive ion reflector mode. Measurements were externally calibrated with $[M+H]^+$ ions of angiotensin II, angiotensin I, substance P, bombesin, and adrenocorticotrophic hormones (clip 1–17 and clip 18–39). Mass spectra were acquired and analyzed automatically using Bruker software, but, if necessary, peak picking and calibration were improved manually using XMASS/NT 5.1.5 (Bruker Daltonics). Database searches were performed by an in-house SWALL sequence database (Swiss-Prot and TrEMBL) using the MASCOT 1.8 software (Matrix Science, London, UK) via BioTools 2.1 software (Bruker Daltonics). A mass tolerance of 80 ppm and one missing cleavage site were allowed, oxidation of methionine residues was considered as variable modification, and carbamido-methylation of cysteines as fixed modification. The search was restricted to human pro-

teins. All results were examined for reliability and occurrence of multiple proteins in the same sample. With a few exceptions, the identification was based on at least five matching peptides. If the database search against the SWALL sequence database resulted in homologous proteins to which the same number of matched peptides was assigned, the entry from Swiss-Prot was selected. Entries from TrEMBL were used only, if no homologous protein was contained in Swiss-Prot or if a higher number of peptides matched to a sequence from the TrEMBL database.

2.8 MALDI-TOF-MS/MS analysis

MALDI-TOF MS/MS measurement of spotted peptide solutions was carried using a 4700 Proteomics-Analyzer (Applied Biosystems). The spectra were recorded in reflector mode in a mass range from 800 to 3700 Da with a focus mass of 2000 Da. If the autolytic fragment of trypsin with the mono-isotopic $[M+H]^+$ m/z at 2211.104 reached S/N of at least 10, an internal calibration was automatically performed using this peak for one-point-calibration. Calibration was performed manually for the less than 1% samples for which the automatic calibration failed. Additionally, MALDI-TOF-TOF analysis was performed for the two strongest peaks of the TOF-spectrum after subtraction of peaks corresponding to background or trypsin fragments. The internal calibration was automatically performed as one-point-calibration if the mono-isotopic arginine $[M+H]^+$ m/z at 175.119 or lysine $[M+H]^+$ m/z at 147.107 reached S/N of at least 5. After calibration, a combined database search of MS and MS/MS measurements was performed using the GPS Explorer software (Applied Biosystems) with the following settings: (i) MS peak filtering: mass range from 800 to 3700 Da; minimum S/N filter of 10; peak density of 50 peaks per range of 200 Da and maximal 200 peaks per protein spot; mass exclusion list contained background peaks and trypsin fragments with an exclusion tolerance of 100 ppm; (ii) MS/MS peak filtering: mass range from 60 Da to a mass that was 20 Da lower than the precursor mass; peak density of 50 peaks per 200 Da and maximal 65 peaks per MS/MS; minimum S/N filter of 10; (iii) database search: precursor tolerance 35 ppm and MS/MS fragment tolerance 0.65 Da.

The peptide search tolerance was 35 ppm but the actual RMS value was between 5 and 15 ppm. Peak lists were compared with the Swiss-Prot or NCBI database restricted to human taxonomy using the MASCOT search engine (Matrix Science). Peptide mixtures that yielded at least twice a MOWSE score [30] of at least 49 for Swiss-Prot and 63 for NCBI results, respectively, were defined as positive identifications.

2.9 Western blotting

Cell lysates were adjusted to 10 mg/mL protein in $1 \times$ Laemmli-buffer. Twenty (for peroxiredoxin 1 and trans-gelatin) or fifty (for peroxiredoxin 4 and proliferating cell

nuclear antigen, PCNA) micrograms, respectively, of total protein extract of each sample was separated by SDS/PAGE electrophoresis (120 V for 2 h) using a Criterion™ Cell (Bio-Rad). Proteins were transferred to NC (Hybond ECL, Amersham Biosciences, UK) by semidry blotting (Bio-Rad) using transfer buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol).

Membranes were blocked for 1 h using Top-Block blocking buffer (FLUKA, Steinheim, Germany) and incubated with antibodies against β -actin (mouse β -actin mAb, Sigma, A-5441, 1:1000; rabbit β -actin polyclonal antibody, DB070, Delta Biolabs, Campbell, USA, 1:200), peroxiredoxin 1 (rabbit anti-peroxiredoxin-1 polyclonal antibody, 16805, 1:2000), peroxiredoxin 4 (mouse anti-peroxiredoxin-4 mAb, 16943, 1:2000), PCNA (rabbit anti-PCNA polyclonal antibody, 2426, 1:200) and transgelin-2 (goat anti-SM22, polyclonal antibody, 10135, 1:1000), all from Abcam (Cambridge, UK). The following secondary antibodies were used: Alexa Fluor 680 goat anti-rabbit IgG, Alexa Fluor 680 rabbit anti-goat IgG (both from Molecular Probes, purchased from Invitrogen, and all diluted 1:10 000), and IRDye 800CW conjugated affinity purified goat-anti-mouse and rabbit-anti-mouse IgG (Rockland, BIOMOL, Hamburg, Germany; 1:5000).

Subsequently, the membranes were washed four times in PBS containing 0.1% Tween 20 and specific signals were detected using an Odyssey® Infrared Imaging System (LICOR Bioscience, Bad Homburg, Germany) according to the manufacturer's instructions. As molecular weight marker, the prestained pEqGOLD marker IV (PEQLAB, Erlangen, Germany) was used. All immunoblots were scanned at a wavelength of 700 nm for Alexa Fluor 680-labeled antibodies and at a wavelength of 800 nm for IRDye 800CW-labeled antibodies. The intensity of the signals was quantified with the Odyssey software version 1.2 using the level of expression of β -actin as internal standard.

2.10 Bioinformatics analysis of protein functions

GeneOntology entries were retrieved based on the XML data files from Uniprot, Genbank and H-InvDB. GeneOntology terms were then mapped to Ontoglyph terms (www.blueprint.org/products/ontoglyphs/) to provide a coarse-grained classification of gene function.

3 Results and discussion

3.1 Proteome map of ReNcell VM NSC

Crude protein extracts from proliferating and differentiating cells of the ReNcell VM cell line were separated by high-resolution 2-DE. A fusion gel of the combined images is displayed in Fig. 1. Each spot detected by the Delta2D software was assigned a unique number to identify spots in a gel matching process (Fig. 1). Methodical replicates of silver-stained gels of the same culture showed high reproducibility

(>95%) by comparison using Delta2D software. As a first step of the 2-DE proteomic approach to the elucidation of this stem cell lines' differentiation program, we created a reference master gel. Spots were isolated and identified from CBB-stained gels of proliferating cells and 4 or 7 days after initiation of differentiation in order to generate a 2-DE-proteome map of ReNcell VM NSC.

Using the 2-DE technology, 956 spots were mapped and 402 spots out of them were identified (Suppl. Table 1). They contained 318 unique proteins. The majority of protein spots were isolated and identified from a single Coomassie gel of proliferating cells. When excising spots from gels of differentiated cells (4 and 7 days) we focused on isolation of up-regulated protein-spots. However, some down-regulated or unregulated spots were also excised for replicate MS identifications. Multiple MS identifications appear in Suppl. Table 1 by double or multiple MS or MS/MS entries and the neighboring lane indicates the corresponding MOWSE scores.

The positions of identified spots are displayed by accession numbers on a fused gel image in Fig. 1. Although the majority of identified proteins (253) were found in only 1 spot, the remaining proteins were detected as multiple isoforms or modifications: 32 proteins appeared in 2 spots, 18 in 3 spots, 5 in 4 spots and 5 proteins were found in 5 spots. Higher numbers of spots with identical proteins were found for the following single proteins: cytoplasmic actin 1 (in 7 spots), creatine kinase B chain (in 8 spots), alpha enolase (in 13 spots), GFAP (in 14 spots) and vimentin (in 15 spots).

To classify identified proteins, 21 functional categories were established based on information from Gene Ontology database and additional information from ExPasy (<http://www.expasy.org/sprot/>). About 21% of the proteins were related to protein synthesis, metabolism, processing and degradation, followed by the groups of cytoskeleton proteins, stress response proteins, the functional group of RNA and other nucleic acids metabolisms and transport (and nuclear proteins), signal transduction and others, each of which did not comprise more than 11% of total identified proteins (Fig. 2).

Until now, human fetal NSC have not been subjected to detailed (2-DE) proteome analysis. First proteomics studies of neuronal differentiation using 2-DE gel methodology have been reported 1999, when Pearce and Svendsen [31] published a short communication about their first differential 2-DE gel approaches of EGF and fibroblast growth factor treated fetal human brain cells. Guo and coworkers [32] identified 24 differentially expressed proteins from retinoic acid treated human embryonic stem cells.

Maurer and coworkers [20] could identify proteins from a 2-DE analysis of neuronal stem cells isolated from adult rat hippocampus. They were able to identify 266 proteins, which they assigned to 12 categories, of which the largest was the metabolism group, which included glycolysis, tricarboxylic acid cycle, amino acid metabolism and protein synthesis. In that study the next largest categories comprised folding,

Table 1. Differentially expressed proteins of ReNcell VM cells of 0 days compared to 4 and 7 days of differentiation

| Protein name | Spot no. | Expres- sion | Accession no. (Swiss- Prot, NCBI) | Theor. MW (kDa) | Theor. pI | MALDI- MS:MOWSE- score | MALDI-MS/ MS:MOWSE- score | Additional proteins in spot | Identical, not regulated proteins |
|--|----------|-----------------|---|-----------------------|--------------|------------------------------|---------------------------------|-----------------------------------|--|
| Cell cycle, cell division | | | | | | | | | |
| Nuclear migration protein nudC | 942 | up | Q9Y266 | 38243 | 5,3 | 86 | | | |
| DNA polymerase delta subunit 2 | 355 | up | P49005 | 51289 | 5,4 | 135 | | 2 | |
| Proliferating cell nuclear antigen (PCNA) | 650 | down | P12004 | 28769 | 4,6 | 171 | 130 | | |
| Microtubule-associated prot. RP/EB family member 1 | 699 | down | Q15691 | 29868 | 5,0 | 83 | 96 | | |
| Nucleosome assembly protein 1-like 1 | 8 | down | P55209 | 45374 | 4,4 | 115 | 126 | | |
| Transcription | | | | | | | | | |
| Cellular nucleic acid binding protein | 947 | down | P62633 | 19462 | 8,0 | 115 | | | |
| | 949 | down | | | | 115 | | | |
| DNA-directed RNA polymerase II 23 kDa polypeptide | 802 | down | P19388 | 24710 | 5,7 | 140 | | | |
| RNA/other nucleic acids metabolism and transport, nuclear proteins | | | | | | | | | |
| Nucleoporin 50 kDa | 934 | up | Q9UKX7 | 50144 | 6,6 | 91 | | | |
| Nucleoporin p54 | 298 | up | Q7Z3B4 | 55435 | 6,5 | 128 | | | |
| Deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial precursor | 886 | down | P33316 | 19346 | 9,7 | 136 | | | |
| Heterogeneous nuclear ribonucleoprotein K | 252 | down | P61978, gi 55958544 | 50976 | 5,4 | 83 | 97 | 1 | 3 |
| Protein synthesis/metabolism/processing/degradation | | | | | | | | | |
| Peptidyl-prolyl cis-trans isomerase B precursor | 35 | up | P23284 | 20289 | 9,3 | 81 | 87 | | |
| Cathepsin D precursor | 747 | up | P07339 | 44552 | 6,1 | 96 | | | |
| Elongation factor 2 (C-terminal fragment) | 378 | up | P13639 | 95207 | 6,4 | 108 | | | 2 |
| Elongation factor 1-delta | 611 | down | P29692 | 30991 | 4,9 | 96 | | | 1 |
| Eukaryotic translation initiation factor 3 subunit 2 | 571 | down | Q13347 | 36502 | 5,4 | 170 | 205, 98 | | |
| Eukaryotic translation initiation factor 3 subunit 4 | 473 | down | O75821 | 35611 | 5,9 | 103 | | | |
| Ubiquilin 1 | 206 | down | Q9UMX0 | 62519 | 5,0 | 135 | | | |
| Proteasome activator complex subunit 3 | 694 | down | P61289 | 29506 | 5,7 | 200 | | | |
| UNR protein | 92 | down | O75534 | 88885 | 5,9 | 97 | | | |
| Nascent-polypeptide-associated complex alpha polypeptide | 18 | down | gi 5031931 | 23370 | 4,5 | | 86 | | |
| Signal transduction | | | | | | | | | |
| Serine-threonine kinase receptor-associated protein | 518 | down | Q9Y3F4, gi 4519417 | 38438 | 5,0 | 107 | 141, 149, 126 | | |
| Stress response | | | | | | | | | |
| Stress-70 protein, mitochondrial precursor | 163 | up | P38646 | 68759 | 5,9 | 93 | 128 | | 2 |
| Heat-shock protein 105 kDa | 61 | up | Q92598 | 96865 | 5,3 | 114, 109 | | 1 | 1 |
| Peroxiredoxin 1 | 848 | up | Q06830 | 22110 | 8,3 | 189 | | | 2 |
| Peroxiredoxin 4 | 785 | down | Q13162 | 30540 | 5,9 | 107 | 65 | | |
| Heat shock 70 kDa protein 4 | 62 | down | P34932 | 94300 | 5,2 | 82 | | 1 | |
| Heat shock cognate 71 kDa protein | 172 | down | P11142 | 70854 | 5,4 | | 84 | | 3 |
| Heat shock protein HSP 90-alpha | 94 | down | P07900 | 84543 | 4,9 | | 163 | 2 | 1 |
| | 101 | down | | | | 134 | | | |
| | 67 | down | | | | | 198 | | |
| Energy metabolism | | | | | | | | | |
| Creatine kinase, B chain | 419 | up | P12277 | 42644 | 5,3 | | 104 | 1 | 7 |
| Bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase, mitochondrial prec. | 624 | down | P13995 | 34138 | 8,9 | 99 | | | |
| Carbohydrate metabolism/transport | | | | | | | | | |
| Aldehyde dehydrogenase X, mitochondrial precursor | 343 | up | P30837 | 55292 | 6,4 | 91 | | | |

Table 1. Continued

| Protein name | Spot no. | Expres- sion | Accession no. (Swiss- Prot, NCBI) | Theor. MW (kDa) | Theor. pI | MALDI- MS:MOWSE- score | MALDI-MS/ MS:MOWSE- score | Additional proteins in spot | Identical, not regulated proteins |
|---|----------|-----------------|---|-----------------------|--------------|------------------------------|---------------------------------|-----------------------------------|--|
| Cytoskeleton | | | | | | | | | |
| Glial fibrillary acidic protein, astrocyte | 355 | up | P14136 | 49880 | 5,4 | | 387 | 2 | 10 |
| | 385 | up | | | | | 511 | | |
| | 418 | up | | | | | 210 | | |
| | 325 | up | | | | | 115 | | |
| Actin, cytoplasmic 1 | 61 | up | P60709 | 41606 | 5,3 | 73, 49 | 105 | 1 | 5 |
| | 418 | up | | | | | 1 | | |
| Actin, cytoplasmic 2 | 355 | up | P63261 | 41662 | 5,3 | 82 | | 2 | 3 |
| Transgelin-2 | 866 | up | P37802 | 22260 | 8,4 | | 130 | | 1 |
| Ezrin (C-terminal fragment) | 419 | up | P15311 | 69268 | 6,0 | 51 | | 1 | 1 |
| Tubulin beta-2 chain (N-terminal fragment) | 600 | up | P07437 | 49671 | 4,8 | 123 | | | 2 |
| Tubulin alpha-6 chain | 570 | up | Q9BQE3 | 49895 | 5,0 | | 107, 95 | | 1 |
| | 252 | down | | | | | 104 | | |
| | 94 | down | | | | | 128 | | |
| | 136 | down | | | | | 135 | | |
| Tubulin alpha-3 chain | 177 | down | Q71U36 | 50136 | 4,9 | 69 | 91 | 1 | 2 |
| | 227 | down | | | | | 208 | | |
| Tubulin alpha-2 chain | 178 | down | Q13748 | 49960 | 5,0 | 64 | | 2 | |
| Vimentin | 340 | up | P08670 | 53520 | 5,1 | 90 | 332 | | 8 |
| | 62 | down | | | | | 68 | | |
| | 94 | down | | | | | 103 | | |
| | 132 | down | | | | | 296 | | |
| | 178 | down | | | | | 100 | | |
| | 227 | down | | | | | 61 | | |
| | 136 | down | | | | | 50 | | |
| Lamin B1 | 177 | down | P20700 | 66277 | 5,1 | 113 | | 1 | 1 |
| | 178 | down | | | | | 154 | | |
| Miscellaneous | | | | | | | | | |
| SET protein (Phosphatase 2A inhibitor I2PP2A) | 15 | down | Q01105 | 33489 | 4,2 | 113 | | | 1 |
| Nuclear autoantigenic sperm protein | 240 | down | P49321 | 85238 | 4,3 | 121 | | | |

started to differentiate into neuronal and glial cells as shown by the phase contrast microscopy. These changes in morphology were linked to significant variations in the protein profile of the ReNcell VM cells (Fig. 3B and C). For the calculation of the expression profiles, normalized fusion images of each two silver-stained gels were compared by Delta2D-software after spot editing. The cellular material of each gel pair derived from either proliferating cells or cells that had been differentiated for 4 and 7 days, respectively. There were minor differences found between the differentiated cells from days 4 and 7, respectively, as compared to the proliferating cells. We intended to solely elucidate expression differences between proliferating and differentiated cells in general thereby minimizing the rate of false positively identified dif-

ferentiation-related proteins. Therefore, we only considered the expression pattern of protein changes if the extent of change varied more than 1.5-fold both 4 and 7 days after start of differentiation when compared to the proliferating control cells (time 0). Applying these criteria, we noted 146 protein spots out of 956 protein spots that displayed differentiation associated changes in their intensities. Seventy-seven spots were down-regulated after 4 and 7 days, respectively, of ReNcell VM differentiation. Thirty of these proteins were identified from the Coomassie-stained gels by MS. Sixty-nine spots were up-regulated after 4 and 7 days of cell differentiation and 19 out of these could be identified. Table 1 shows the differentially expressed proteins being identified, sorted according to protein function and up- or down-regulation.

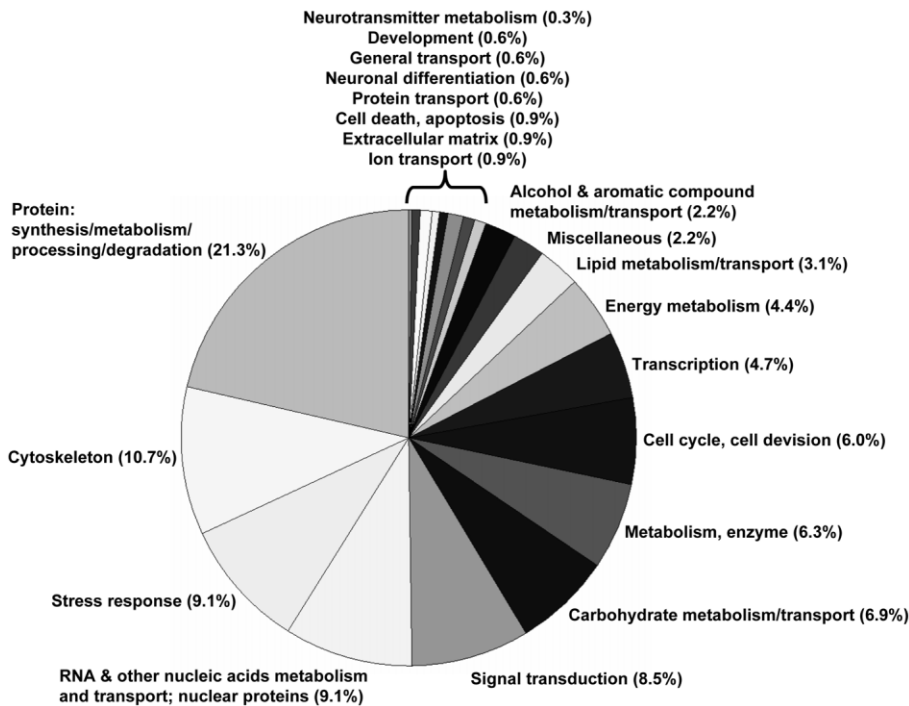


Figure 2. Relative quantitative distribution of functional protein categories. The 21 functional protein categories are based on $n = 318$ individual proteins and are largely assigned according to gene ontology database. The largest group (protein synthesis, metabolism, processing and degradation) comprises 21% of the proteins, whereas the proportions of the remaining contributors do not exceed 11%.

To validate the results of the proteome analysis, quantitative Western blot experiments were performed for two up-regulated (transgelin-2 and peroxiredoxin 1) and two down-regulated (peroxiredoxin 4 and PCNA) proteins (Fig. 4).

PCNA protein [44, 45] was strongly down-regulated according to 2-DE analysis (spot 650) and the Western blot analysis revealed reduction of the protein levels by 60 and 80% after 4 and 7 days of differentiation, respectively. Originally, this protein was described as an autoantibody to a nuclear antigen in proliferating cells [44] and further characterized as a protein correlating to the proliferative state of cells [46]. PCNA as well as other cycle-associated proteins turned out to be common markers of proliferating cells [47]. PCNA interacts with multiple partners mainly involved in DNA replication, DNA repair and cell cycle control and other cellular events like maintenance of chromosome structure [48]. One well-described interacting partner out of the maintenance group is the chromatin assembly factor 1 (CAF-1). We also identified the subunit C of this factor in spot 337, but it did not display any differentiation-dependent regulations of its level. CAF-1 is a molecular chaperone that deposits histones H3 and H4 onto newly replicated DNA, while PCNA topologically marks newly replicated DNA for chromatin assembly by CAF-1 [49]. Using a proteomic approach, Hengstschläger and coworkers [50] have recently demonstrated that the expression of PCNA and the p48 (= C) subunit of CAF-1 is subject to the regulation by the tuberous sclerosis gene products (TSC1 and TSC2). Ectopic over-expression of these proteins in HeLa cells triggered the up-regulation of the expression of PCNA and CAF-1. The strong

down-regulation of PCNA in differentiating ReNcell VM cells is in line with the stop of proliferation of the cells after growth factor withdrawal.

The expression level of two out of four peroxiredoxins that were identified on our 2-DE gels, *i.e.* peroxiredoxin 1 (PRDX1) and 4 (PRDX4), was changed significantly during differentiation of ReNcell VM cells. For the two remaining peroxiredoxins, PRDX 2 and 6, we could not detect a significant up- or down-regulation.

PRDX1 was allocated to three spots. During the course of the differentiation all three spots displayed increased intensities but only one spot (spot 848) exceeded the cut-off level of 1.5-fold both after 4 and 7 days of differentiation. Our Western blot analysis confirmed an almost 2-fold increase of the PRDX1 amount during differentiation of the cells. PRDX4 was found only in spot 785 and the initial 2-DE-based observation of a strong down-regulation in differentiated ReNcell VM cells was supported by the Western blot results that displayed a more than 2-fold down-regulation of this redox regulation protein after 4 and 7 days of differentiation.

The peroxiredoxins are a family of anti-oxidative proteins, consisting of six members in mammals. They are distributed in the cytosol, mitochondria, peroxisomes and plasma. Besides their role as a peroxidases some members show further functions, *e.g.* in cell proliferation, differentiation and gene expression, presumably independent on their peroxidase activity [51, 52]. Hirotsu and coworkers [53] associated different isoforms of almost identical proteins from human, rat and mouse with diverse cellular functions, such

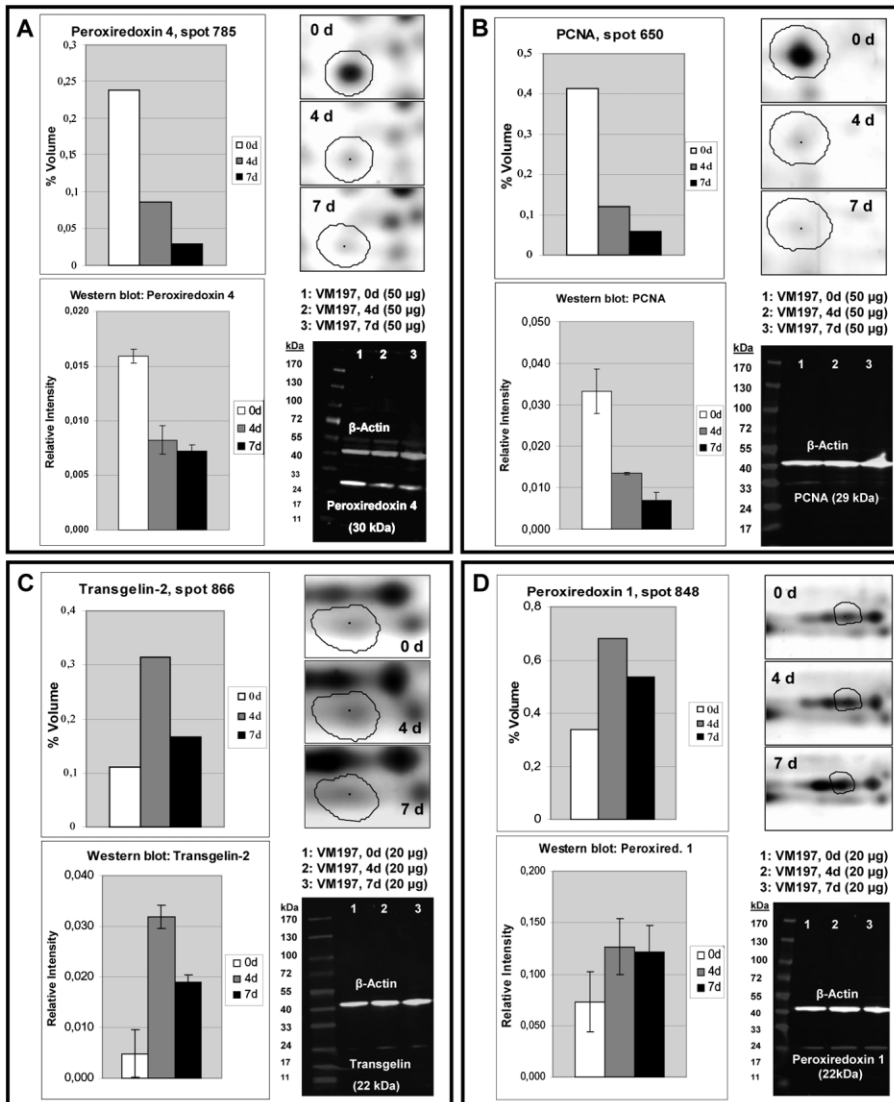


Figure 4. Examples of differentially regulated proteins of differentiating ReNcell VM cells. Quantitative analysis of 2-DE gel separated proteins is compared with results of quantitative Western blot analysis (normalized to beta-actin as internal standard) for the proteins peroxiredoxin 4 (A), PCNA (B), transgelin-2 (C) and peroxiredoxin 1 (D). For technical details of Western blot analysis see Section 2.

Transgelin-2 (TAGL2), a protein of the calponin family, was identified in spot 866, whose intensity increased 3-fold after 4 days differentiation of ReNcell VM cells and 1.7-fold after 7 days. Furthermore, transgelin-2 was identified in spots 51 and 855 but the level of these spots displayed only smaller differentiation-associated increases. Supporting the results of the proteome analysis, the Western blot analysis revealed a strong up-regulation after 4 days (6.4-fold), which was slightly diminished after 7 days (3.8-fold) of cell differentiation.

Transgelin interacts directly with actin filaments; saturation of transgelin-binding occurs at a transgelin/actin monomers ratio of 1 to 6, which causes their rapid gelation within minutes [60]. Three human isoforms of transgelin can be found in the UniProtKB/Swiss-Prot database: TAGL (transgelin, smooth muscle protein 22-alpha, SM22-alpha),

TAGL2 (transgelin-2, SM22-alpha homolog) and TAGL3 (transgelin-3, neuronal protein NP25, neuronal protein 22). TAGL and TAGL3 are 64 or 69%, respectively, identical to TAGL2. The SM22- α protein (transgelin-2) [61, 62] is described as early marker of smooth muscle cell gene expression during vascular/cardiac development [63]. Nevertheless, its exact role in this context has not been yet clarified. Some factors, *e.g.* PDGF (platelet-derived growth factor), AVP (arginine vasopressin), myocardin and Arp2/3 have been shown to influence SM-22 expression in smooth muscle cells [64–68]. In rat colonic epithelial cells and fibroblast it has been shown that transgelin level is associated with Ras activation [69].

Although it is still necessary to further characterize this actin interacting protein, the strong early up-regulation of TAGL2 in differentiating ReNcell VM cells (and less inten-

sive TAGL3) and the linkage of transgelin to the RAS signaling pathway suggests this protein to be an interesting candidate for further investigations of early neuronal development.

Many of the other identified proteins, which are differentially expressed after transition from proliferation to differentiation, are not yet identified to play an active role in the process of differentiation. However, the following proteins, representing a large spectrum of functional groups, may be interesting candidates for further elucidation of their participation in the neural differentiation process such as stop of cell division and induction of apoptosis, establishing of cell adherence junctions, growth cone development, neurite elongation and synapse formation.

The nucleosome assembly protein 1-like 2 protein (NP1L2) controls the neurulation according to investigations of Rogner and coworkers [70] in a mutant mouse model. Defects were correlated with an overproduction of neuronal precursor cells. We found the 55% homologous NP1L1-protein out of the NAP-1 gene family to be accordingly down-regulated in the differentiated ReNcell VM cells. Two proteins controlling cell cycle and cell division, *i.e.* the nuclear migration protein nudC that is known to be involved in neurogenesis and neuronal migration [71, 72] and the DNA polymerase delta subunit 2, are increased during differentiation in our study, which is in accordance with published data [72, 73].

The heterogeneous nuclear ribonucleoprotein K represses the translation of p21, an enhancer of neuronal differentiation, in non-neuronal and neuronal cells [74] and was found to be down-regulated in our study. Nucleoporin 50 kDa (Nup50) is a component of the nuclear pore complex with a role in protein export, and its expression increased in differentiated ReNcell VM cells compared to proliferating cells in our 2-DE expression approach. Nup50 was shown before to be strongly expressed in developing neural tubes, and its disruption resulted in neural tube defects [75].

Two proteins of the functional group of protein synthesis/metabolism/processing/degradation, *i.e.* ubiquilin and the elongation factor 2, are known to be involved in neuronal development. Ubiquilin-1 (synonym: PLIC-1) that protects against neuronal apoptosis [76] and counteracts neuronal acetylcholine receptors [77] is down-regulated in differentiated ReNcell VM cells in our study. Furthermore, ubiquilin-1 links CD47 (integrin-associated protein, IAP) to the cytoskeleton via vimentin [78] and regulates the surface expression of GABA-A receptors [79]. In addition, PDI (protein-disulfide isomerase) that is up-regulated in response to hypoxia/brain ischemia in astrocytes interacts with ubiquilin [80]. The elongation factor 2 increases protein synthesis involved in neuronal differentiation of N1E115 mouse neuroblastoma cells [81] and is up-regulated in differentiated ReNcell VM cells in our approach.

Creatine kinases are involved in energy metabolism of cells and tissues with high-energy demands such as brain [82]. Creatine kinase B chain (BCK) protein expression was

increased in the differentiated ReNcell VM cells. BCK mRNA level was reported to be strongly expressed in primary neurons of 1-day-old zebrafish embryos and it is suggested to play role in neuronal differentiation [83].

There are five stress-related proteins besides the above-mentioned peroxiredoxins found to be differentially regulated in our study. The up-regulated stress-70 protein (GRP75, 75 kDa glucose regulated protein) is implicated in the control of cell proliferation and cellular aging [84, 85]. The heat shock protein HSP-105 was also up-regulated, whereas HSP-70, HSP 90-alpha and HSP7C were down-regulated. The relationship of these heat shock proteins to the process of neural differentiation remains unclear. However, these findings may reflect different functions of distinct members of the heat shock protein family for differentiation processes, which are certainly connected with cellular stress.

Generally, reassembling of cytoskeletal proteins is an important feature of morphological changes during neural differentiation [86, 87]. Therefore, it is not surprising that more than 10 out of 45 differentially regulated proteins belong to components of microfilaments, microtubuli and intermediary filaments. The group of up-regulated cytoskeleton proteins includes actin (cytoplasmatic 1 and 2), ezrin (a C-terminal fragment), tubulins and four GFAP (astrocyte). In total GFAP was identified alone or together with other proteins in further 14 spots. Of those, 4 (325, 355, 385 and 418) displayed a differentiation-associated increase in intensity. For spots 325 and 385 this increase is clearly due to higher GFAP levels, because both spots contained only GFAP. GFAP is a well-known cell-specific marker [88] that, during the development of the central nervous system, distinguishes astrocytes from other glial cells. The up-regulation of GFAP in a number of spots confirms the expected 50% differentiation of ReNcell VM stem cells into glia, mainly astrocytes using the most simple differentiation procedure. The extensive structural rearrangements of the cytoskeleton are reflected by changes in the intensity of spots containing structure proteins. In addition to some tubulin and laminin spots, predominantly vimentin was down-regulated in six spots (and once up-regulated). The intermediate filament vimentin is used as (immature) radial glial marker [89–91]. However, only certain isoforms of vimentin displayed reduced levels in differentiating ReNcell VM stem cells because eight additional vimentin spots did not display significant changes in their intensities during the time course of the experiment. Concerning the other differentially regulated proteins, the current literature is lacking evidence for a direct involvement in neural differentiation processes. Nevertheless, they may contribute to these processes in a so far not disclosed manner.

The comparison of the results of our approach with those of proteomics screening of Maurer and coworkers [11] in NSC from adult rat hippocampus shows a few corresponding protein expressions. The proteins regulated concordantly to our study are the elongation factor 2 (up-regulated in 9 of 11 spots), tubulin alpha (down-regulated in 2 of 6 spots) and

β -actin (= cytoplasmic 1, up-regulated in 1 of 2 spots). A reverse expression was found for peroxiredoxin 1 and 4: peroxiredoxin 4 was up-regulated and peroxiredoxin 1 down-regulated (each in a single spot) during differentiation in the study of Maurer and coworkers [11]. The few congruent results between studies on fetal rat midbrain and adult human hippocampal stem cells indicate a lack of common features of NSC of different origin with regard to proteome profiling, which is also supported by gene expression microarray studies in different systems [6–9, 55].

4 Concluding remarks

In the present study, we describe a proteome database of hNSC isolated from the ventral mesencephalon and immortalized via *v-myc*. This database provides a protein inventory that will allow specifying changes in the protein expression pattern due to specific pathways activated or suppressed during proliferation and differentiation of NSC. Therefore, the present database is not only a valuable basis for short-term interval assessment of differentiation-related changes of protein expression but also presents the possibility to employ the expression pattern observed as a quality control during the cultivation and differentiation process of hNSC. Furthermore, it will help to describe the pathways, which are involved in differentiation and neural plasticity, indicating a functional role for newly identified proteins in hNSC.

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