Proteomics Analysis of Epithelial Cells Reprogrammed in Cell-free Extract*

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The functional reprogramming of a differentiated cell to a pluripotent state presents potential beneficial applications in regenerative medicine. We report here the proteomic profile of 293T epithelial cells reprogrammed to a pluripotent state using undifferentiated embryonal carcinoma (NCCIT) cellular extracts. 293T cells were reversibly permeabilized with streptolysin O, incubated in an extract of NCCIT cells or a control extract of 293T cells for 1 h, resealed with CaCl2, and cultured. OCT4 and SOX2 gene expression were up-regulated in NCCIT extract-treated cells relative to control cells, whereas there was no alteration in DNMT3B gene expression. Thirty percent of NCCIT extract-treated cells were positive for SSEA-4, and karyotyping confirmed their 293T origin, excluding the possibility of contamination from NCCIT cells. Two-dimensional PAGE revealed ~400 protein spots for each cell type studied. At least 10 protein spots in the proteome of NCCIT extract-treated cells had an expression profile similar to that of NCCIT and remained unaltered in control cells. Using tandem mass spectrometry, we identified these proteins, which include 78-kDa glucose-regulated protein precursor and tropomyosin α-3 chain. This investigation provides the first evidence that proteins are altered in a specific manner in NCCIT extract-treated cells. This is the first report on the proteomic characterization of the nuclear reprogramming process. Molecular & Cellular Proteomics 8:1401–1412, 2009.

The reprogramming of terminally differentiated somatic cells into an undifferentiated state has recently become a major research focus (1–3). Successful nuclear reprogramming (NR) has great potential in the field of regenerative medicine. For example, NR may facilitate the generation of isogenic replacement cells for the treatment of a variety of diseases while overcoming many of the ethical issues raised by the use of embryonic fetal cells (4).

The search for factors that facilitate the reprogramming of differentiated cells has been underway for at least 2 decades. Among the various approaches that have been used to achieve and investigate reprogramming, somatic cell nuclear transfer (5–8), cellular fusion (9, 10), and the use of undifferentiated EC cellular extracts (11) have all been shown to lead to the reversion of the donor genome to a less differentiated state. The rationale behind these methodologies is that a host cell (or extract) provides all the necessary regulatory components that mediate alterations in the gene expression and protein expression of the target genome. One of the most exciting advances in NR technology has been achieved within the last 2 years ultimately with the generation of induced pluripotent stem cells (12–16). The method involves the retroviral introduction of four defined transcription factors, Oct-4/Sox2/c-myc/Klf4 (13, 15) or Oct-4/Sox2/Nanog/Lin28 (17), into somatic cells, which is sufficient to reprogram them into embryonic-like stem cells.

Despite the simplicity of most reprogramming technologies, such as the induced pluripotent stem cell approach, they prove to be very inefficient. Moreover understanding of the reprogramming process is in its infancy. Little is known about how various reprogramming factors actively confer pluripotency upon the somatic cell nucleus. Individual chromatin-remodeling factors (13, 18), DNA and histone modifications (11, 13, 14), and alterations in gene expression (11, 13, 14) have been implicated. However, to date there is no information regarding the global protein alterations occurring following the induction of reprogramming. Characterizing the changes in the reprogrammed cell proteome will provide a more expansive view of events occurring during reprogramming and help identify candidate proteins involved in maintaining pluripotency long after the initial induction events have occurred.

The abbreviations used are: NR, nuclear reprogramming; GRP78, 78-kDa glucose-regulated protein; SAKS1, stress-activated protein kinase substrate protein 1; SSEA, stage-specific embryonic antigen; SLO, streptolysin O; TPM3, tropomyosin α-3 chain; TRA, battle of Trafalgar; NCCIT, undifferentiated embryonal carcinoma; EC, embryonal carcinoma; 2D, two-dimensional; Nex, NCCIT extract; 293Tex, 293T extract; HBSS, Hank’s balanced salt solution; FACS, fluorescence-activated cell sorting.

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Proteomic Profile of Reprogrammed Cells

Here we report the characterization of the proteomic profile of human embryonic kidney epithelial cells subjected to a reprogramming protocol using undifferentiated embryonic carcinoma cell extract. For the first time, we show an embryonic cell surface antigen pattern associated with cells reprogrammed using extracts. Additionally using two-dimensional (2D) PAGE we identified a number of proteins altered in direct response to the reprogramming protocol. These proteins display an expression profile similar to that of embryonic carcinoma cells. We speculate that these proteins are altered as a consequence of the reprogramming protocol and are involved in maintaining the pluripotent state.

EXPERIMENTAL PROCEDURES

Cell Culture—293T cells (human embryonic kidney epithelial cells) were grown to ~70% confluency at 37 °C and 5% CO2 in complete culture medium containing Dulbecco’s modified Eagle’s medium (Sigma) with 10% FCS (Invitrogen), 2 mM l-glutamine (Sigma), 1 mM sodium pyruvate (Invitrogen), and nonessential amino acids (Sigma). 293T cells treated with NCCIT (Nex) or 293T extract (293Tex) were seeded at 100,000 cells/well in a 48-well plate and cultured in 250 μl of complete RPMI 1640 medium with antibiotics. The Nex sample group consisted of two biological replicates of 293T cells treated with an NCCIT cell extract.

Cell Extracts—To prepare NCCIT extracts, cells were washed in PBS and in cell lysis buffer (100 mM HEPES, pH 8.2, 50 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, protease inhibitors (all Sigma), sedimented at 400 × g, resuspended in 1 volume of cold cell lysis buffer, and incubated for 30–45 min on ice. Cells were sonicated on ice in culture for a minimum of 2 weeks prior to further experimentation. After sonication, the cell extract was incubated at 400 × g, resuspended in 1 volume of cold cell lysis buffer, and incubated for 30–45 min on ice. Cells were sonicated on ice in 200-μl aliquots using a pulse sonicator fitted with a 3-mm-diameter probe until all cells and nuclei were lysed as judged by microscopy. The lysate was sedimented at 15,000 × g for 15 min at 4 °C to pellet the coarse material. The supernatant was aliquotted, frozen in liquid nitrogen, and stored at −80 °C.

SLO-mediated Permeabilization and Cell Extract Treatment—293T cells were washed in cold PBS and in cold Ca2+- and Mg2+-free Hanks’ balanced salt solution (HBSS) (Invitrogen). Cells were resuspended in aliquots of 100,000 cells/100 μl of HBSS, placed in 1.5-ml tubes, and centrifuged at 120 × g for 5 min at 4 °C in a swing-out rotor. Sedimented cells were suspended in 97.7 μl of cold HBSS, tubes were placed in a H2O bath at 37 °C for 2 min, and 2.3 μl of SLO (Sigma) (100 μg/ml stock diluted 1:10 in cold HBSS) was added to a final SLO concentration of 230 ng/ml. Samples were incubated horizontally in a H2O bath for 50 min at 37 °C with occasional agitation and set on ice. Samples were diluted with 200 μl of cold HBSS, and cells were sedimented at 120 × g for 5 min at 4 °C. Permeabilization was measured by monitoring uptake of a 70,000 Mw, Texas Red-conjugated dextran (50 μg/ml; Invitrogen) in a separate sample 24 h after resealing and replating the cells. Permeabilization efficiency under these conditions was ~80%.

After permeabilization, cells were suspended at 1000 cells/μl in 100 μl of NCCIT (n = 2) or 293T (control) (n = 2) extract containing an ATP-regenerating system (1 mM ATP, 10 mM creatine phosphate, 25 μg/ml creatine kinase (all Sigma), 100 μM GTP (Sigma), and 1 mM each nucleotide triphosphate (NTP; Roche Diagnostics). The tube containing cells was incubated horizontally for 1 h at 37 °C in a H2O bath with occasional agitation. To resell plasma membranes, the extract was diluted with complete RPMI 1640 medium containing 2 mM CaCl2 and antibiotics, and cells were seeded at 100,000 cells/well on a 48-well plate. After 2 h, floating cells were removed, and plated cells were cultured in complete RPMI 1640 medium. Nex and 293Tex samples were maintained in culture for a minimum of 2 weeks prior to further experimentation.

RNA Isolation, cDNA Synthesis, and Quantitative PCR—Total RNA was extracted from 293T, NCCIT, Nex, and 293Tex after 12 weeks of culture using TRI Reagent following the manufacturer’s guidelines (Sigma). Total RNA was treated with DNase I (DNA-free kit, Ambion, Huntingdon, UK) to remove any DNA contamination from samples. First strand cDNA synthesis was performed using oligo(dT) primers (Metabion, Martinsried, Germany) and the Superscript II reverse transcriptase system (Invitrogen).

To verify whether the reprogramming protocol caused alterations in relative gene expression quantitative real time PCR was performed on an iCycler (Bio-Rad) using the prepared cDNA and primers for OCT4, SOX2, DNMT3B, and human β-actin for normalization. All experiments included negative controls with no cDNA. SYBR Green Jump Start Master Mix (Sigma) was added to each well of the plate (10 μl of SYBR Green, 7 μl of RNase free water, 2 μl of forward and reverse primers, and 1 μl of cDNA). The PCR primers used were as follows (5’→3’): OCT4 forward, GGA AGG TAT TCA GCC AAA CGA CCA; OCT4 reverse, CTC ACT CGG TTC TCG ATG TGT; SOX2 forward, TTC GAT GGC AAC TTT CTA T; SOX2 reverse, ACA TGA ATT CTC GCC AGA C; DNMT3B forward, AGA TCA AGG TCG TCA AT; DNMT3B reverse, GAC ATG TGG GCT TTA GGA A; ACTB forward, CAA GAT CAT TGC TCC TCC TG; and ACTB reverse, ATC CAC ATC TGC TGG AAG. Quantitative PCR was also used to confirm the changes in protein expression witnessed by 2D PAGE. The relative gene expression of 78-kDa glucose-regulated protein (GRP78), stress-activated protein kinase substrate protein 1 (SASK1), and TPM3 was measured. The PCR primers used were as follows (5’→3’): GRP78 forward, AGG ACA AGA AGG AGG ACG TG; GRP78 reverse, TTG GAG GTG ATG TGC TTA TT; SASK1 forward, GGA AGG AGC GGC AGT TG; SASK1 reverse, TCT GCT TGC TCC CTC TCG AT; TPM3 forward, CTG CAG GAG GAG CTG AAG AA; and TPM3 reverse, CAG CTT GGC TAG CCA TCT CT. PCR was performed under the following conditions: 50 cycles in total; 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s cycles with a start time of 95 °C for 3 min. Samples were run in triplicate. Data were analyzed using iCycler (Bio-Rad) software. Gene expression data for OCT4, SOX2, DNMT3B, SASK1, GRP78, and TPM3 was normalized against β-actin using the “ΔΔCt method” to give an expression value relative to the internal control of β-actin expression (19). Statistical analysis was performed by using one-way analysis of variance with Tukey’s multiple comparison test. p < 0.05 was considered significant.

Karyotyping—The cytogenetic analysis was performed using standard G-banding techniques. Cells cultured in a T25 flask were treated with 0.1 μg/ml Colcemid (Invitrogen) for up to 4 h followed by dissociation with trypsin/verseine. The cells were pelleted via centrifugation, resuspended in prewarmed 0.0375 M KCl hypotonic solution, and incubated for 10 min. Following centrifugation, the cells were resuspended in fixative (3:1 methanol:acetic acid). Metaphase spreads were prepared on glass microscope slides, G-banded by brief exposure to trypsin, and stained with 4:1 Gurr’s/Leishmann’s stain. A total of 30 metaphase cells were examined for each culture. Flow Cytometric Analysis—FACS analysis was carried out as described previously (20). Briefly 293T, NCCIT, Nex (n = 2), and 293Tex (n = 2) cells were removed from culture flasks by mechanical disassociation. Cells were centrifuged at 300 × g for 5 min and washed twice in wash buffer (10% (v/v) fetal calf serum + PBS). The cell pellet was resuspended at 105 cells/ml. 5 × 106 cells were incubated with SSEA-1, SSEA-3, SSEA-4, TRA1-60, TRA1-81, TRA2-49/E, or TRA2-54 hybridoma supernatant for 30 min at 4 °C. The cells were washed twice in wash buffer before resuspension in wash buffer containing FITC-conjugated goat anti-mouse IgG + IgM (Caltag, Buckingham, UK) at a final dilution of 1:100. Samples were incubated for 30 min at 4 °C. The cells were washed a final time in wash buffer,
and cell pellets were resuspended in 500 μl of wash buffer containing 10 μg/ml propidium iodide. The samples were analyzed on a Cy-AnADP flow cytometer (Dako, Glostrup, Denmark). A minimum of 10,000 viable cells were collected for investigation. Analysis was undertaken using a 20-milliwatt, 488 nm solid state laser, cells were identified by light scatter (forward versus side), and live/dead cell discrimination was performed by propidium iodide exclusion, measured with a dichroic long pass and a 613/20 band pass filter. FITC immunofluorescence was measured using a 545 dichroic long pass and a 530/40 band pass filter. The data were analyzed using Summit 4.3 software (Dako).

2D PAGE—293T, NCCIT, Nex (n = 2), and 293Tex (n = 2) cells were removed from culture flasks by mechanical dissociation. Cells were washed twice in PBS (without Ca2+ and Mg2+) and then resuspended in 1 ml of Buffer A (8 M urea, 2% (w/v) CHAPS, protease inhibitors). The resulting samples were then diluted further using Buffer A by a 1:7 ratio. This was done to reduce the viscosity of the samples. A Plus-one 2D cleanup kit (GE Healthcare) was used according to the manufacturer’s instructions to purify, desalt, and remove all impurities from the protein samples. The resulting protein pellet was redissolved in Buffer A. The bicinchoninic acid (BCA) assay was performed as described previously (21) to determine the protein concentration of all samples. Briefly 10 μl of each protein sample was added to 200 μl of 2% (v/v) copper sulfate solution in BCA and incubated at 37 °C in the dark for 30 min. Absorbance was read at 570 nm using a Benchmark 96-well plate reader (Bio-Rad).

Protein samples were diluted to a concentration of 1.7 μg of protein/μl in Buffer A after which 0.5% (v/v) IPG buffer pH 4–7 (GE Healthcare) and 0.002% (w/v) bromophenol blue were added. DTT (Sigma) was then added to give a final concentration of 40 mM DTT. 300 μg of protein was used to rehydrate 18-cm, pH 4–7 IPG strips (GE Healthcare). Proteins were resolved in the first dimension by IEF for a total of 27,000 V-h using the IPGphor isoelectric focusing system (GE Healthcare). Proteins were resolved for 12 h at room temperature. Each extraction was followed by centrifugation and removal of supernatants. The original supernatant and the supernatants from the three sequential extractions were pooled and dried in a vacuum centrifuge for 4–6 h. The dried peptides were dissolved in 7 μl of 0.1% (v/v) formic acid in 3% (v/v) ACN in water. Samples were centrifuged for 5 min at 12,000 × g, and the supernatants were subjected to LC-ESI-MS/MS.

LC-ESI-MS/MS—LC separations of the tryptic digests were performed using a Dionex Ultimate 3000 variable flow nano-LC system (Dionex, LC Packings). Sample peptides were injected, initially transferred, and desalted by a PepMap C18 microguard column (300-μm internal diameter × 1 mm) (LC-Dionex, Leeds, UK) using transfer buffer A. Samples were subsequently eluted to an analytical column (PepMap C18; 75-μm internal diameter × 15-cm column (LC-Dionex)) by elution buffer B. The elution process was extended across a 30-min gradient with a linear ramp from 5 to 50% elution buffer B. The compositions of transfer buffer A and elution buffer B were 5% (v/v) ACN, 0.1% (v/v) formic acid and 95% (v/v) ACN, 0.1% (v/v) formic acid, respectively. The column eluents were transferred via fused silica to a nano-ESI source, sprayed directly into a QSTAR XL Hybrid ESI-Q-TOF mass spectrometer (Applied Biosystems, MDS-Sciex) and analyzed over tandem MS. An initial MS survey scan was performed over a transmission range of 300–1800 m/z. Two dynamically excluded peptide precursor ions with charges +2 and +3 of the highest intensity were then selected for CID tandem MS. Dissociated ions were ejected to complete a second pass MS2 scan at a maximum transmission range of 75–200 m/z. Time-of-flight accumulation times for MS and MS2 were set at 1 and 3 s, respectively. Scans and data acquisition software were operated using Analyst QS v1.1 (Applied Biosystems, MDS-Sciex) on a multi-node work station.

LC-ESI-MS/MS Data Analysis—Spectral data from LC-ESI-MS/MS analysis were searched against the Swiss-Prot protein database (Swiss-Prot 54.4, February 11, 2007, 287,050 entries) using a sequence query search by MASCOT software (version 2.4). Presubmission peak list conversion was done using the Applied Biosystems wiff-mgf filter via Mascot.dll v1.6 release 1.9 (Matrix Science, Boston, MA) using default parameters. All spectra were deconvoluted at both the MS and MS/MS level prior to MASCOT search. The taxonomy filter string was limited to Homo sapiens databases to reduce cross-species redundancy. Tryptsin was used as the cleavage enzyme with an allowed two missed cleavages. The precursor mass (MS) tolerance was set at 1.2 Da, and fragment mass (MS/MS) tolerance was set at 0.6 Da. Carbamidomethyl modification of cysteine and oxidized methionine were set as variable modifications. MASCOT protein matches were considered valid if ion scores satisfied one of the following criteria: identification with at least one top ranking peptide with a MASCOT score of more than 51 (p < 0.001) or at least two top ranking peptides each with a MASCOT score of more than 33 (p <
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0.05). MS/MS queries matching multiple protein homologues were assigned to the homologue with at least one top ranking (red and bold) peptide of score higher than 51. Previously assigned peptides (red, non-bold peptides) were considered as peptides belonging to another hit.

Verification of Proteins Identified by 2D PAGE Using Western Blot Analysis—To verify the identity of proteins identified by 2D PAGE, 10 μg of protein from 293T, NCCIT, Nex, and 293Tex was separated by SDS-PAGE using 4–20% precast polyacrylamide gels (Perbio Science UK Ltd., Northumberland, UK). Gels were run at 25 mA for ~3 h using a Mini-Protean II™ gel electrophoresis system (Bio-Rad). Resolved proteins were transferred to a PVDF Immobilon PSQ transfer membrane (0.2-μm pore size) (Millipore, Watford, UK) using a Bio-Rad Mini Trans-blot electrophoretic transfer cell. Following transfer, membranes were blocked with 2% (w/v) nonfat milk powder in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TTBS) for 1 h at room temperature. Blocked membranes were then incubated with either of the following antibodies: mouse anti-BiP/GRP78 monoclonal antibody (catalogue number 610978, BD Biosciences) or mouse anti-β-actin monoclonal antibody (catalogue number A8826, Abcam, Cambridge, UK). Antibodies were diluted 1:250 and 1:1000, respectively, in 1% (w/v) milk powder in TTBS and incubated overnight at 4 °C. After four washes with TTBS, membranes were incubated in a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (catalogue number 554002, BD Biosciences) for 1.5 h (1:1000 dilution). After four more washes with TTBS, immunoreactive proteins on the membranes were detected using SuperSignal West Dura chemiluminescent reagents (Perbio) and exposure to Hyperfilm ECL high performance chemiluminescent film (GE Healthcare).

Experimental Design—Four groups of cells were used in experiments: NCCIT, 293T, and 293T cells treated with either Nex or 293Tex groups. Two biological replicates were pooled for both Nex and 293Tex groups. The level of OCT4, SOX2, and DNMT3B expression was measured in all groups using quantitative PCR. Cytogenetic analysis of the cells in all four experimental groups was conducted by karyotyping. This was done to confirm that all samples subjected to the reprogramming protocol were of 293T origin. FACS analysis was also performed to study the expression of embryonic cell surface antigens in all groups. Experiments were designed to compare the proteomic profile of 293T cells subjected to a reprogramming protocol with NCCIT and 293T cell extracts. Biological replication in addition to technical replication of gels was performed. The different extract treatments were compared with each other and NCCIT and 293T cells. Any protein spots in 2D gels displaying greater than ±1.5-fold alteration in expression were considered as being altered. These proteins were then selected for further investigation using LC-ESI-MS/MS. To verify the proteins identified by 2D PAGE, Western blotting was carried out as described above. For further insights into mechanisms, quantitative PCR analyses were also conducted.

RESULTS

NCCIT Extract-treated Cells Up-regulate Genes and Proteins Associated with Pluripotency—293T cells treated with Nex or 293Tex were compared with 293T and NCCIT phenotypes. The quantitative expression profiles of OCT4, SOX2, and DNMT3B in all four experimental samples is shown in Fig. 1. The levels of OCT4 (p < 0.05) and SOX2 (p < 0.05) in Nex were elevated by ~20- and ~2-fold, respectively, relative to expression in 293T cells not subjected to the reprogramming procedure. DNMT3B expression in Nex was not significantly altered when compared with 293T cells. None of the pluripotency markers tested showed altered expression in the control, 293Tex (Fig. 1). Furthermore, cytogenetic analysis confirmed that 293T, Nex, and 293Tex cells were of 293T origin, and none of the examined Nex cells showed an NCCIT karyotype (see supplemental Table S1).

NCCIT, 293T, Nex, and 293Tex samples were then tested for the presence of the embryonic cell surface antigens SSEA-1, SSEA-3, SSEA-4, TRA1-60, TRA1-81, TRA1-85, TRA2-49, and TRA2-54 using FACS. The NCCIT cells expressed a cell surface antigen pattern associated with EC cell lines; i.e. SSEA-3, SSEA-4, TRA1-60, TRA1-81, TRA1-85, TRA2-49, and TRA2-54 were all detected, whereas SSEA-1 expression was absent (supplemental Fig. S1). In contrast,
Nex cells were positive for SSEA-4, a marker of pluripotency, with ~30% of Nex cells expressing SSEA-4 (Fig. 2). Nex cells did not express any of the other cell surface antigens associated with pluripotency. All cell lines were positive for TRA1-85, which recognizes a cell surface antigen present in almost all human cells (22).

**2D Gel Analysis of the Proteome in Extract-treated Cells**—Over 400 protein spots were visualized in each of the three gel replicates. The proteomes of Nex, 293Tex, 293T, and NCCIT cells were compared. Detailed information on the similarities and differences across the four proteomic profiles is provided in supplemental Fig. S2. As four different experimental samples were being cross-compared, a set of guidelines was established to allow several comparisons to be made. The proteomic profiles of Nex and 293Tex were compared with 293T and NCCIT proteomic profiles, respectively. Those proteins whose expression levels were the same as those in 293T and NCCIT were categorized as group A. Proteins that were

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**Fig. 2. Surface antigen marker expression.** Using FACS analysis the expression of surface antigen markers characteristic of undifferentiated (EC and embryonic stem) cells was examined (for details regarding all antigen markers examined see supplemental Fig. S1). Here we show expression data for the pluripotency marker SSEA-4 together with the negative and positive controls, P3X and TRA1-85, respectively (y axis represents cell count, and x axis represents the FITC intensity). Approximately 30% of Nex cells were positive for SSEA-4.
the same as those in 293T but were altered in expression when compared with NCCIT cells were categorized as group B. Those altered compared with 293T but regarded as having the same expression profile as those in NCCIT were classed as group C. Finally those proteins that had an altered expression profile when compared with both 293T and NCCIT were placed in group D (Tables I and II). A second comparable analysis was conducted to compare the proteomic profiles of 293Tex with 293T and NCCIT. Like groups A–D, their proteins were divided into groups A–D, respectively (Tables I and II).

To compare Nex with 293Tex, subgroups A–D and A′–D′ (see Tables I and II) were compared with each other using Venn diagrams. One comparison of particular interest is that of subgroup C with subgroup C′. This revealed 12 protein spots that had an expression similar to that in NCCIT. Of these, 10 protein spots showed different expression when compared with 293Tex. These 10 proteins are probably involved in conferring the reprogramming process in the Nex cells.

**Fig. 3.** Venn diagrams depicting the common and different proteins between proteomes. To compare Nex with 293Tex, subgroups A–D and A′–D′ (see Tables I and II) were compared with each other using Venn diagrams. One comparison of particular interest is that of subgroup C with subgroup C′. This revealed 12 protein spots that had an expression similar to that in NCCIT. Of these, 10 protein spots showed different expression when compared with 293Tex. These 10 proteins are probably involved in conferring the reprogramming process in the Nex cells.

**Validation of Observed Protein Expression Profiles**—To confirm some of the changes in protein expression, we performed Western blot analysis using antibodies against GRP78 and β-actin (loading control) to show that the results confirmed the 2D PAGE findings (Fig. 6). We also performed real time RT-PCR analysis to examine the gene expression profiles of a number of differentially expressed proteins in extract-treated cells. Real time RT-PCR was performed on the four experimental samples and normalized against the β-actin housekeeping gene (Fig. 7). Although many of Nex and 293Tex gene expression profiles for GRP78, SAKS1, and TPM3 replicated the protein expression profiles obtained from 2D PAGE, some of the mRNA expression patterns did not correlate with the protein expression profiles.

**DISCUSSION**

Despite the development of new methods for reprogramming somatic cells to pluripotency, little is understood about the molecular mechanisms involved in reprogramming beyond a handful of genes. In this study we characterized global proteomic changes associated with nuclear reprogramming. We subjected epithelial cells to a reprogramming protocol.

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**TABLE I**

| Subgroup | 293T | NCCIT | Number of spots |
|----------|------|-------|-----------------|
| A        | Same | Same  | 210             |
| B        | Same | Altered| 22              |
| C        | Altered | Same | 12              |
| D        | Altered | Altered | 21              |

**TABLE II**

| Subgroup | 293T | NCCIT | Number of spots |
|----------|------|-------|-----------------|
| A′       | Same | Same  | 193             |
| B′       | Same | Altered| 7               |
| C′       | Altered | Same | 11              |
| D′       | Altered | Altered | 54              |
using undifferentiated NCCIT cell extracts and characterized the extent of reprogramming, cell surface antigen profile, and changes to the proteomic profile in these cells.

First we measured the extent to which Nex cells were reprogrammed. Previous investigations by Freberg et al. (24) demonstrated that extracts derived from NCCIT EC cells can reprogram 293T nuclei eliciting epigenetic reprogramming of OCT4 by targeted demethylation, resulting in increased expression of OCT4 in reprogrammed cells (24). We confirm here the overexpression of OCT4 in NCCIT extract-treated cells, although up-regulation of SOX2 was more modest in these experiments (Fig. 1). Further in contrast to findings by Taranger et al. (11), our study revealed no up-regulation of DNMT3B. These differences may be explained by the time lag between reprogramming initiation and assessment of gene expression profiles. Taranger et al. (11) confirmed the up-regulation of OCT4, SOX2, and DNMT3B at weeks 2, 4, and 6 after reprogramming. The Nex cells used in this study were subjected to the reprogramming protocol 12 weeks prior to RNA extraction and protein preparation because of the requirement for high cell numbers for proteomics analysis. These differences were anticipated considering the number of studies that have shown temporal changes in target cell gene expression as a result of using cellular extracts to reprogram cells (18, 25, 26).

SSEA-4 is a stage-specific embryonic antigen present on the surface of embryonic germ, carcinoma, and stem cells (27) and was induced in Nex cells. Notably during retinoic acid differentiation, embryonic carcinoma cells such as NCCIT continue to express SSEA-4 for longer than other pluripotency markers (28). We speculate that in the process of nuclear reprogramming SSEA-4 is one of the first pluripotency markers to become activated and expressed (Fig. 8). The fact that SSEA-4 was the only cell surface pluripotency marker expressed in Nex cells suggests the occurrence of partial reprogramming, which has been described previously (24, 29). Further investigation is required to ascertain whether a prolonged exposure or repeated exposures to the NCCIT extract together with an earlier assessment of embryonic cell surface antigen expression would show any changes in the expression of other surface markers associated with pluripotency. Notably cytogenetic analyses confirmed that increased OCT4 and SOX2 gene expression and SSEA-4 protein expression were not the result of residual intact NCCIT cells carried over from the nuclear reprogramming protocol (supplemental Fig. S2).

To date, research has focused on characterizing changes in gene expression in reprogrammed cells (11, 24). A limitation of transcriptomics approaches is that the functional significance of any changes in gene expression remains unknown because mRNA expression does not always reflect protein expression: even at a steady state, post-transcriptional, translational, or post-translational regulation during protein synthesis often results in potentially large discrepancies between protein and mRNA expression (30–34). We applied high resolution 2D PAGE and tandem mass spectrometry to show that
a number of proteins in Nex followed a pattern of expression similar to that in the NCCIT profile (group C proteins; Table III). Most of these proteins were identified as protein- and nucleotide-binding proteins. It is therefore likely that these proteins play a role in events associated with alterations of chromatin, histone post-translational modifications, and DNA modifications occurring during reprogramming.

Among these, GRP78 and TPM3 expression was increased in Nex cells when compared with 293T cells. GRP78 (P11021) is a member of the heat shock protein 70 (HSP70) family that functions as a chaperone critical for folding, maturation, and transport of polypeptides and peptides (35). Expression of GRP78 is highly elevated in many cancers, correlating with malignancy and metastasis, and has been implicated in signaling pathways that lead to proproliferative and antiapoptotic behaviors (35–39). Similarly we found that the protein TPM3 (P06753) was up-regulated in Nex cells. TPM3 is a cytoskeletal protein involved in cell motility and cytoskeletal organization (40). Up-regulation of TPM3 is associated with an aggressive tumor phenotype, increased cell mobility, and suppression of differentiation (41). Our finding of GRP78 and
TPM3 up-regulation in Nex cells suggests the direct involvement of these proteins in NR. Both of these proteins have established roles in cancer cells and have been implicated in pathways conferring behaviors associated with pluripotency (antiapoptotic and differentiation suppression). We speculate that similar pathways are involved in NR, and further investigations are required to test this hypothesis.

SAKS1 expression was also elevated in Nex and NCCIT cells when compared with 293T cells. SAKS1 is a scaffolding protein that enhances the unfolding and proteolytic digestion of a subset of proteins by the proteosome (42). It is only possible to speculate on why SAKS1 was up-regulated in Nex cells. One interesting hypothesis is that SAKS1 may aid in the proteolytic destruction of proteins characteristic of 293T cells. In this way, particular proteins that are present within a differentiated cell to confer its cell fate may be actively degraded in the establishment of reprogramming. To our knowledge, this is the first time a model of active degradation of host proteins has been proposed as a mechanism of NR. This may well provide an explanation for how NR is able to be rapidly established within the protein environment of a differentiated cell. This hypothesis requires further investigation.

The persistence of widespread proteomic changes within the host cell proteome 12 weeks after initial reprogramming induction suggests a fundamental role for these factors in NR. It is currently not possible to conclude whether the up-regulated expression of proteins identified here is rapidly induced by initial reprogramming events or occurs downstream of initial events. Further investigations can ascertain the extent to which these factors are required to establish reprogramming and the extent to which these proteins are causative or facilitative. Of note, we found a number of proteins that were altered solely as a result of incubating 293T cells in a 293T extract. This result was anticipated from the findings of Taranger et al. (11) who showed that a proportion of genes becomes up-regulated as a result of such a treatment. Thus it may be that 293T cells initiated a reprogramming-unrelated response to permeabilization and/or extract treatment per se.

The examination of the proteomic profile of the cells 12 weeks after reprogramming was because of the requirement for large quantities of cultured cells to produce three replicates gels with 300 μg of protein loaded onto each gel. Although resolving a high number of proteins, 2D PAGE has

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**Fig. 7.** mRNA expression of identified proteins GRP78, SAKS1, and TPM3. The expression of GRP78 in Nex when compared with 293Tex replicates the pattern of protein expression within those samples as does the pattern of expression of GRP78 in NCCIT when compared with Nex; this is also statistically significant (p < 0.05). The mRNA expression does not correlate with the protein expression when other samples are compared. Studying the mRNA and protein expression profile of SAKS1 expression across the experimental samples shows that when Nex and 293Tex are compared the mRNA and protein profiles correlate. This is the same for TPM3 expression. In all other comparisons of SAKS1 and TPM3 expression there is no correlation between mRNA and protein expression. The expression values were normalized based on β-actin expression values. Graphs display mean value, and error bars display the S.E. * and # indicate statistical significance between groups.

**Fig. 8.** Model of cell surface antigen expression during reprogramming using NCCIT cell extracts. SSEA-4 expression persists for longer during differentiation of embryonic carcinoma cells exposed to retinoic acid (45). SSEA-4 expression was the only marker to become expressed following reprogramming using Nex. This suggests that what is observed here is partial reprogramming.


**TABLE III**

The proteomic profile of 293T, Nex, 293Tex, and NCCIT

Subgroup A encompasses proteins that show the same expression in all four experimental samples, subgroup B represents proteins in Nex cells that show the same expression in 293T cells, subgroup C contains proteins in Nex cells whose expression is altered from that seen in the 293T proteomic profile but are characteristic of the NCCIT proteomic profile, and subgroup D encompasses proteins in Nex cells whose expression is altered and uncharacteristic of both the 293T and NCCIT proteomic profiles. Also shown are the molecular function and biological process of the proteins, UniProt accession number, theoretical molecular mass (Da), pI, and molecular weight search (MOWSE) score. A full summary of all the identified proteins is provided in supplemental Table S2, and Q-TOF spectral data are presented in supplemental Table S2. See supplemental Table S2 for full details of MS/MS queries leading to protein identifications, and GO annotation assignments. SAPK, stress-activated protein kinase.

| Predicted protein                                      | Subgroup | Molecular function                                                                 | Biological process                          | Accession no. | Molecular mass | pI       | Unique peptides | Coverage | MOWSE score |
|--------------------------------------------------------|----------|------------------------------------------------------------------------------------|---------------------------------------------|---------------|----------------|---------|----------------|----------|-------------|
| Elongation factor 2                                     | A        | Nucleic acid, nucleotide, and protein binding; hydrolase activity                 | Metabolic process                          | P13639        | 95,277         | 6.41    | 3              | 3        | 73          |
| N^2,N^2-Dimethylarginine dimethylaminohydrolase 1       | A        | Ion binding, hydrolase activity                                                   | Cell communication, metabolic process       | O94760        | 31,102         | 5.53    | 6              | 24       | 191         |
| Pyruvate kinase isozymes M1/M2                          | A        | Ion and protein binding, transferase activity                                   | Metabolic process                          | P14618        | 57,900         | 7.96    | 2              | 5        | 103         |
| Ras GTPase-activating protein-binding protein 1         | B        | Protein binding                                                                   | Metabolic process                          | Q13283        | 52,132         | 5.36    | 2              | 6        | 125         |
| Protein-disulfide isomerase A3 precursor                | B        | Protein binding                                                                   | Metabolic process                          | P30101        | 56,747         | 5.98    | 14             | 37       | 596         |
| Mannose 6-phosphate receptor-binding protein 1          | B        | Protein binding                                                                   | Cell localization                          | O60664        | 47,018         | 5.3     | 5              | 17       | 337         |
| Pyruvate dehydrogenase E1 component subunit β, mitochondrial precursor | B        | Oxido-reductase activity                                                          | Metabolic process                          | P11177        | 39,208         | 6.2     | 6              | 19       | 230         |
| Cu,Zn-superoxide dismutase                             | B        | Ion binding, oxido-reductase activity                                             | Anatomical structure formation, stress response | P00441        | 15,926         | 5.7     | 2              | 16       | 146         |
| 78-kDa glucose-regulated protein precursor             | C        | Nucleotide and protein binding                                                   | Cell localization                          | P11021        | 72,288         | 5.07    | 10             | 38       | 798         |
| Heat shock cognate 71-kDa protein                      | C        | Protein binding                                                                   | Metabolic process                          | P11142        | 70,854         | 5.37    | 12             | 26       | 382         |
| SAPK substrate protein 1                               | C        | Protein binding                                                                   | Cell localization                          | Q04323        | 33,305         | 5.23    | 3              | 19       | 139         |
| Tropomyosin α-3 chain                                  | C        | Protein binding                                                                   | Cell motility, cytoskeletal                | P06753        | 32,799         | 4.68    | 2              | 8        | 58          |
| Hsc70-interacting protein                              | D        | Protein binding                                                                   | Metabolic process                          | P50502        | 41,305         | 5.18    | 6              | 18       | 347         |
| Peroxiredoxin-6                                       | D        | Peroxidase, hydrolase, and oxido-reductase activity                              | Metabolic process, stress response         | P30041        | 25,019         | 6       | 10             | 42       | 386         |
| 26 S protease regulatory subunit 6B                    | D        | Nucleotide and protein binding, hydrolase activity                              | Metabolic process                          | P43686        | 47,337         | 5.09    | 11             | 36       | 490         |
technical drawbacks, including difficulties in detecting low abundance, very high molecular weight, or very low molecular weight proteins. These are therefore typically underrepresented on a 2D gel; thus, it was difficult to focus on less abundant molecules, such as OCT4. Further experiments involving purification of nuclear proteins in Nex cells would provide greater insight into alterations occurring within the nuclear proteome during NR. With respect to the future of regenerative medicine and directed differentiation, nuclear expression proteomics offers insights as to what nuclear profile is needed to program or reprogram cellular fate with limited imprinting side effects (43). Additionally to nuclear expression proteomics, quantification of post-translational modifications of histones and other proteins occurring during expression proteomics, quantification of post-translational modifications of histones and other proteins occurring during NR will undoubtedly reveal key molecular events. To this end, more sensitive methods, such as isobaric tags for relative and absolute quantitation (TTRAQ), are required. This stable isotope labeling technique is advantageous as it provides more accurate quantitation than the use of 2D gels (44), smaller amounts of protein (<100 μg/phenotype) are required, and it would allow the measurement of NR alterations over time.

In conclusion, this is the first systemic proteomics analysis of cells undergoing nuclear reprogramming. This investigation has identified a number of proteins that are altered in a specific manner in NCCI/T extract–treated cells. We speculate that these identified proteins have associated roles in maintaining/confering the pluripotent state in reprogrammed cells.

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