Quantitative Proteomics Analysis Demonstrates Post-transcriptional Regulation of Embryonic Stem Cell Differentiation to Hematopoiesis

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Embryonic stem (ES) cells can differentiate in vitro to produce the endothelial and hematopoietic precursor, the hemangioblasts, which are derived from the mesoderm germ layer. Differentiation of Brachyury (Bry)ES cells to hemangioblasts can be followed by the expression of the Flk1 and Flk1 genes. Proteomic and transcriptomic changes during this differentiation process were analyzed to identify mechanisms for phenotypic change during early differentiation. Three populations of differentiating BryES cells were obtained by flow cytometric sorting, GFP^− Flk1^− (epiblast), GFP^+ Flk1^− (mesoderm), and GFP^+ Flk1^+ (hemangioblast). Microarray analyses and relative quantification two-dimensional LCLC-MS/MS on nuclear extracts were performed. We identified and quantified 2389 proteins, 1057 of which were associated to their microarray probe set. These included a variety of low abundance transcription factors, e.g., UTF1, Sox2, Oct4, and E2F4, demonstrating a high level of proteomic penetrance. When paired comparisons of changes in the mRNA and protein expression levels were performed low levels of correlation were found. A strong correlation between isobaric tag-derived relative quantification and Western blot analysis was found for a number of nuclear proteins. Pathway and ontology analysis identified proteins known to be involved in the regulation of stem cell differentiation, and proteins with no described function in early ES cell development were also shown to change markedly at the proteome level only. ES cell development is regulated at the mRNA and protein level.

Molecular & Cellular Proteomics 7:459–472, 2008.

In the mouse embryo the process of gastrulation generates the mesodermal layer. During embry development the mesoderm is generated from the epiblast or embryonic ectoderm.

The cells involved in mesodermal commitment show a marked increase in Brachyury (Bry) expression (1). The mesoderm migrates and develops into tissues including the endothelium as well as blood (2, 3). A common progenitor for these cell types, the hemangioblast, is first detected at the midstreak stage of gastrulation (4). These processes of early cell development within the embryo are not easily assessed because of limitations on the material available for study. This can be overcome by using embryonic stem (ES) cell lines derived from the inner cell mass of the blastocyst-stage embryo (5). ES cells can be allowed to differentiate in vitro to form three-dimensional spheroid cultures known as embryoid bodies (EBs). These structures contain derivatives of all three germ cell layers: mesoderm, endoderm, and ectoderm (6, 7). Mesoderm-derived populations contained within the EBs include hematopoietic and endothelial progeny. This model has been likened to the temporal pattern of development of these populations in embryogenesis (8–10), and EB cultures have been shown to contain cells with hemangioblast potential (11, 12).

Differentiating ES cells in vitro with the use of the EB model system have been valuable in identifying a number of genes associated with self-renewal, such as Oct4, Sox2, and Nanog (13–16). These proteins are considered as markers for the pluripotent embryonic stem cell in mouse and human (13, 17–20). Disruption of Oct4 or Nanog results in the differentiation of ES cells to trophectoderm and extraembryonic endoderm, respectively (13, 17, 18, 20). The target genes for these proteins often encode transcription factors and chromatin-remodeling enzymes (21). Other genes have key roles in the process of mesoderm and hemangioblast development.

1 The abbreviations used are: Bry, Brachyury; ES, embryonic stem; GFP, green fluorescent protein; Bry^APPP+, ES, GFP has been knocked into one locus of the Brachyury gene, replacing exon 1; EB, embryoid body; Flk1, vascular endothelial growth factor 1 receptor 2; HMG, high mobility group; HDAC, histone deacetylase; DNMT1, DNA methyltransferase 1; iTRAQ, isobaric tags for relative and absolute quantitation; LIF, leukemia inhibitory factor; SOM, self-organizing map; MTG, monothioglycerol; GO, gene ontology; Rb, retinoblastoma.
For example the transcription factors Scl/tal-1 and Runx1 have been shown to function in early development specifically at the stage of hematopoietic commitment (22–26).

Bry is a member of the T-box gene family encoding transcription factors that are thought to be involved in differentiation (27). The majority of the cells contained within the primitive streak are Bry-positive; expression is rapidly downregulated as the cells migrate (1). In vitro Fehling et al. (28) have targeted the Bry locus with green fluorescent protein (GFP) and demonstrated that it was expressed in mesoderm; this was later confirmed to be the case in vivo (4). Flk1, the vascular endothelial growth factor 1 receptor 2, is essential for yolk sac blood island development and vasculogenesis; knockout experiments demonstrated reduced hematopoiesis (29–31). The process of mesodermal precursor cell generation within the primitive streak and migration to the site of yolk sac development is associated with Flk1 expression (31). In differentiating ES cells the expression of Flk1 overlaps that of Bry and the formation of the hemangioblast-like cells (23). It was subsequently shown that identifying Bry and Flk1 expression enabled the enrichment of three populations of cultured ES cells. These represent a premesoderm stage (Bry<sup>−</sup>Flk1<sup>−</sup>), a prehemangioblast mesoderm stage (Bry<sup>−</sup>Flk1<sup>+</sup> cells), and the hemangioblast cell stage (Bry<sup>−</sup>Flk1<sup>−</sup> Flk1<sup>+</sup> cells). Gene expression analysis of the three populations of cells showed striking differences that were linked to the biological potential of the cells. For example two members of the Wnt family, Wnt3a and Wnt8a, whose expression is seen in the primitive streak and early mesoderm, were only seen in GFP<sup>−</sup>Flk1<sup>−</sup> and GFP<sup>+</sup>Flk1<sup>−</sup> cells (28) (also see Fig. 1). Runx1 and Scl were predominantly seen in the GFP<sup>−</sup>Flk1<sup>−</sup> cells, which is consistent with the ability of this population of cells to generate hemangioblast cells (28).

Protein families such as transcription factors and chromatin-remodeling enzymes are known to be the target of post-transcriptional regulation that affects the level of protein, whereas mRNA levels are unchanged (32–35). Using systematic approaches mRNA levels have been shown in several systems not to comprehensively reflect changes in protein levels (36–41). This observation argues that systematic analyses of stem cells and progenitor cells must include proteomics analysis for full details to be acquired of regulatory pathways associated with differentiation.

We have therefore established a method for defining the proteome of stem cell populations using isotopic tags for relative and absolute quantitation (iTRAQ). This allows four experiments to be analyzed simultaneously, giving relative quantification on many hundreds of proteins from a relatively small (1–5 million) cell number. Unwin et al. (42) compared transcriptome changes with those in the proteome using hematopoietic stem cell-enriched populations compared with their differentiated progeny. They demonstrated that 54% of protein changes observed are not seen at the transcriptional level. Thus the process of differentiation is governed at the protein turnover/synthesis level as well as by rates of mRNA synthesis/degradation. To obtain the detail on nuclear protein regulation in enriched populations of primitive cells we used premesoderm stage (Bry<sup>−</sup>Flk1<sup>−</sup>) cells, a prehemangioblast mesoderm stage (Bry<sup>−</sup>Flk1<sup>−</sup>), and hemangioblast stage (Bry<sup>−</sup>Flk1<sup>−</sup> Flk1<sup>+</sup>) cells. These are enriched from differentiating ES cells where GFP is targeted to the Bry locus. This offers an effective marker for the mesodermal population that develops within the EBs. Here we systematically study this progression in development and show that protein expression is controlled at the post-transcriptional and translational levels in developing ES cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture** — The GFP gene was knocked into one allele of the Bry gene of the mouse embryonic stem cell line E14.1 replacing exon 1 as described in Fehling et al. (28) generating the Bry<sup>GFP</sup>/<sup>+</sup> ES cell line. Maintenance and differentiation of these cells have been described previously (28, 43). Briefly ES cells were maintained on irradiated embryonic feeder cells in Dulbecco’s modified Eagle’s medium supplemented with 15% (v/v) FCS, penicillin, streptomycin, LIF (1% (v/v) conditioned medium (44, 45)), and 1.5 \times 10^{-4} M monothioglycerol (MTG; Sigma). Prior to the onset of differentiation the cells were passaged twice on gelatinized plates. The first passage was into Dulbecco’s modified Eagle’s medium supplemented as above, and the second was into Iscove’s modified Dulbecco’s medium supplemented with 15% (v/v) FCS, penicillin, streptomycin, LIF (1% (v/v) conditioned medium), and 1.5 \times 10^{-4} M MTG (Sigma). The ES cell population was harvested at this stage.

For the generation of EBs, ES cells were trypsinized and plated at 2.5 \times 10^{4} cells/ml. Differentiation of EBs was carried out in 100-mm Petri grade dishes in Iscove’s modified Dulbecco’s medium supplemented with 15% (v/v) FCS, 2 mM L-glutamine (Invitrogen), 200 \mu g/ml transferrin, 0.5 mM ascorbic acid (Sigma), and 4.5 \times 10^{-4} M MTG.

**Flow Cytometry and Cell Sorting** — EBs were harvested and trypsinized, and the resultant single cell suspension was analyzed on a FACScalibur flow cytometer (BD Biosciences). Staining and isolation of cell populations were performed as described previously (28). Co-expression of GFP with the receptor tyrosine kinase Flk1 revealed the emergence of three distinct cell populations, GFP<sup>−</sup>Flk1<sup>−</sup>, GFP<sup>−</sup>Flk1<sup>+</sup>, and GFP<sup>+</sup>Flk1<sup>−</sup> Flk1<sup>+</sup> cells, which represent a developmental progression ranging from premesoderm to prehemangioblast mesoderm to the hemangioblast. These sorted populations were used for analysis of the proteome and microarray analysis of transcription. Furthermore the gene expression pattern of each sorted population was assessed to ensure that appropriate cell enrichment had occurred.

**Gene Expression Analysis** — Total RNA was extracted using an RNeasy minikit and treated with RNase-free DNase (Qiagen, Valencia, CA). Total RNA (2 \mu g) was reverse transcribed into cDNA with random hexamers using the Omniscript RT kit (Qiagen). PCR was performed with ~100 ng of cDNA and Biomix Red (Bioline). Cycling conditions were as follows: 95 °C for 3 min followed by 25–35 cycles of amplification (95 °C denaturation for 30 s, annealing for 30 s, and 72 °C elongation for 30 s) with a final incubation at 72 °C for 5 min. Details of primer sequences, annealing temperature, and cycle numbers for each PCR are shown in Supplemental Table 1.

**Real Time PCR** — Approximately 50 ng of cDNA was used with TaqMan Universal PCR Master Mix (Applied Biosystems, Warrington, UK) for all real time PCRs. Cycling conditions were as follows: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Details of probes used can be found in Supplemental Table 2.
Nuclear Fractionation—Nuclear fractions of the ES and sorted differentia
ted cells were obtained with the use of the Nuclear Extract kit
(Active Motif, Brussels, Belgium) as described in the manufacturer’s
instructions. The nuclei were then lysed with 8 m urea. The lysates
were homogenized via passage through a 23-gauge needle and then
dialyzed in 0.9 m urea.

Mass Spectrometry—Protein samples (100 µg) were prepared as
described previously (42), then digested by addition of 5 µg of trypsin
at 0.5 µg/µl, and incubated overnight at 37 °C. Peptides were labeled
with iTRAQ reagent (Applied Biosystems, Framingham, MA) as de
scribed previously (42). Reactions were then checked for complete
labeling using mass spectrometry; the four isobarically tagged sam
ples (named 114, 115, 116, and 117 after the reporter ion associated
with the tag) were then pooled prior to analysis.

Peptide Fractionation and Mass Spectrometry—Prior to reverse
phase LC-MS/MS peptides were fractionated off line using a strong
cation exchange chromatography column (10-cm × 2.1-mm Polyclc
PolySulfoethyl A column, 5-µm beads, 200-Å pore size (The Nest
Group, Southborough, MA) on an LC Packings Ultimate LC system.
The gradient was run at 250 µl/min using initially 10 mM KH2PO4, 20%
ACN, pH 2.7 with KCl concentration increasing from 0 to 250 mM KCl
over a 45-min period and then from 250 to 500 mM over 10 min
followed by 1 M for 5 min. Five-minute fractions were collected for
the first 15 min followed by 1-min fractions. Each fraction was then
concentrated. Reverse phase LC-MS/MS was then performed using
the procedures described previously (42). For analysis, about 30% of
the peptide sample was used on each run with the QSTAR® XL mass
spectrometer (Applied Biosystems, Warrington, UK).

Transcriptome Analysis—RNA from GFP Flk1−, GFP Flk1+, and
GFP Flk1−/H9262 ES cells was prepared in triplicate using the Qiagen
RNeasy kit (Qiagen, Crawley, UK) according to the manufacturer’s
instructions. Transcriptome analysis was undertaken by the Cancer
Research UK microarray facility (Paterson Institute, Manchester, UK).
Details of the protocols are available upon request. Total RNA was
subjected to amplification using the “gene chip eukaryotic small sample
labeling assay version II” developed by Affymetrix, and the gene
expression profile was assessed using Mouse 430-2 chips in triplicate.

Data Analysis—Proteome data were searched against a mouse
KBMS3.0 protein database from Celera Discovery System (369,691
entries) (Applied Biosystems, Framingham, MA) using ProQUANT
version 1.1 (Applied Biosystems) as described previously (42). Default
search parameters within ProQUANT were set with trypsin as the
digestion agent allowing for one missed cleavage, an MS tolerance of
0.15 Da, and an MS/MS tolerance of 0.1 Da. Fixed modifications
included methylmethanethiosulfonate alkylation of cysteine and
iTRAQ modification of lysine and N termini. Variable modifications
included iTRAQ labeling of tyrosine. Only peptides with a minimum
confidence score of 70 were included in the database; this allowed
only the highest quality MS/MS spectra to be used for protein identi
fication and therefore relative quantification. Quantitation was per
formed by comparing iTRAQ reporter ion peak areas where iTRAQ
peaks less than 40 counts were not used. Outliers were not removed.
A minimum of three peptides were required to identify and relatively
quantify a protein. Quantitation was achieved by calculating a
weighted average from all matching peptides along with a 95% confi
dence interval to assess accuracy of protein ratio. Experiments
were performed in triplicate, and the ProGROUP (Applied Biosys
tems) software was used to pool data from all experiments and assign
a single accession number for each protein, allowing comparison of
replicate datasets. iTRAQ ratios greater than or equal to 1.2 or less
than 0.8 found in three experiments with a p value less than 0.05 were
flagged as being differentially expressed. Performing the search
against a concatenated database containing both forward and re
versed sequences (therefore 739,382 entries) allowed estimation of
the false discovery level. Transcriptome data were analyzed by sub
mitting each replicate pair individually to GeneChip® Operating Soft
ware version 1.2 software (Affymetrix) with default settings with the
exception of the target normalization value, which was set to 100. A
transcript was determined to be “significantly differentially ex
pressed” if it was called “present” and “increased” or “decreased” in
all three replicate arrays.

To compare transcriptome and proteome data, all proteins with a
Swiss-Prot, TrEMBL, or RefSeq accession number were selected,
and these accession numbers were used to identify the relevant
probe set identifiers from the microarray dataset using the NetAffx
tool (Affymetrix). The Pathway Analyst software (Stratagene, Amster
dam, The Netherlands) was used to map interaction pathways on
protein changes.

Various self-organizing map (SOM) size matrices were tested with
a 1 × 3 map being the most appropriate for the generation of distinct
cluster groups whose members shared similar expression profiles as
cells differentiated. SOMs were trained using the Kohonen algorithm.
Two training phases were used. Phase 1 (the ordering phase) con
sisted of 2000 epochs, an initial learning rate of 0.5 decreasing to 0.1,
and a neighborhood size of 1. Phase 2 (the fine tuning phase) con
sisted of 1000 epochs, a consistent learning rate of 0.1, and a neigh
borhood size of 0.

Western Blot Analysis—Nuclear fractions and whole cell lysates of
ES cells sorted on the characteristics described above were size-
fractionated by SDS-PAGE, and proteins were transferred onto nitro
cellulose membrane (Hybond-C, Amersham Biosciences) in transfer
buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.0375% SDS)
using an XCell SureLock™ (Invitrogen). Membranes were blocked
with 5% (w/v) nonfat milk solution in TTBS (0.05% Tween 20, 20 mM
Tris-HCl, pH 7.5, 500 mM NaCl) for 2 h. Immobilized antigen was
detected following incubation with primary antibody diluted in 1%
(w/v) nonfat milk solution in TTBS overnight. Blots were then washed
using TTBS and incubated with the appropriate horseradish peroxi
dase-conjugated secondary antibody in 5% (w/v) nonfat milk solution
in TTBS for 1 h. The blots were then washed using TTBS and devel
oped using ECL (Amersham Biosciences). Antibodies and their
sources are shown in Supplemental Table 3.

RESULTS

Selection Based on Flk1 and Brachyury Expression to Gen
erate Cells for Systematic Microarray and Proteomics Analysis of Early Development—When ES cells are cultured in the
absence of LIF, in a medium that supports mesodermal de
velopment, BryGFP+/− ES cells display an ability to differentiate.
Furthermore the presence of Brachyury at a single allele has
been shown to have no adverse effect on development (28).
Thus the system offers opportunities for flow cytometric sort
ing of BryGFP+/− ES cells based on GFP expression enabling
the enrichment of cells at specific stages of development.
After 2.5 days of differentiation in suspension culture a
GFP Flk1− population was sorted using a fluorescence-acti
vated cell sorter. Cells of this phenotype have been shown
previously to have characteristics of epiblast-like cells. At 3.5
days GFP Flk1− (mesoderm-committed cells) and
GFP Flk1− cells (enriched for hemangioblast-like cells, see
Fig. 1A) were sorted, and these three populations along with
unsorted ES cells were used for microarray or proteomics
analysis. Prior to these analyses the developmental stage of
the four populations was assessed at both the genomic and

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biological level. Gene expression analysis was performed on RNA extracted from the cell populations (Fig. 1). The expression of genes known to be expressed in these developing cells was determined. The blast cell colony-forming potential of the sorted cell populations was also evaluated, and as anticipated (28), the majority was seen within the GFP/H11001 Flk1/H11001 cells (data not shown). The markers used for flow cytometric enrichment thus offer opportunity for systematic analysis on developmental progression ranging from premesoderm to prehemangioblast.

Stem cell signatures have been defined at the mRNA level using microarrays (46–48). However, we have shown previously that 54% of proteomic changes in hematopoietic stem cells do not correlate with that seen for specific mRNA species (41). Proteomics technologies require decreased sample complexity to identify lower abundance proteins. We therefore fractionated the BryGFP/BrEES cells, enriching for nuclei. The nuclear fraction contained ~67% of total cellular protein in all four populations. Fig. 1C shows that the marker proteins for cytosol and nucleus reside predominantly in the appropriate fractions. The nuclear fractions from the four populations were each trypsinized and labeled with a specific iTRAQ reagent. The samples were then mixed and subjected to two-dimensional LC-MS/MS. Protein identification and relative quantification was achieved by integrating the peptide data for each specific protein. The protein content per cell was the same in all four populations in all three experiments performed. Eighty-one percent of proteins we identified and quantified had previously been defined with a nuclear localization (49).

The enrichment procedure had the desired effect as peptides derived from low abundance proteins (such as transcription factors, e.g. Sox2 (see Fig. 5)) were detected that have not been detected in similar experiments on whole cell lysates. Identification and relative quantification on 2389 proteins (>3 peptides/protein) was achieved (see Supplemental Table 4). Previous studies have shown cutoff values for significant change at the protein level to be ≥1.2 and ≤0.8 (42, 50–54). Unwin et al. (42) demonstrated that comparison of biological replicates in one iTRAQ experiment enabled accurate differences in expression to be identified at a ratio of ≥1.2 and ≤0.8 for ≥3 peptides. These values have been used as one criterion for proteins that show a change in expression level. To identify differentially expressed proteins a minimum of three peptides were required to identify and relatively quantify a protein. iTRAQ ratios also required a p value less than
0.05 (95% confidence limit of proteins considered to change) for the protein to be considered as changed. Of the four populations of cells included in the analysis the undifferentiated the Bry +/- GFP ES cell population was the only population that was unsorted. It was therefore a highly heterogenous population of cells that also included apoptotic cells. When comparative analysis was performed on this population it was found to give the greatest variation between the triplicate datasets, and as such these cells were excluded from further analysis. The GFP +/- Flk1- cells compared with GFP +/- Flk1- cells exhibited 8.5% of proteins changing significantly (192 proteins) between these two populations. GFP +/- Flk1- cells showed a 14% change (334 proteins) when compared with GFP +/- Flk1- cells. Furthermore GFP +/- Flk1- cells compared with GFP +/- Flk1- cells showed 9.5% of proteins changed significantly in their level of expression (226 proteins). We next determined how this related to changes in transcription from these genes. Searching against a reversed database allowed calculation of the false discovery rate as 0.15962% at the protein level.

Proteomics and Genomics Analysis of Developing Bry/GFP+/+ ES Cells—Further analysis of these data was performed with the comparison of the protein relative quantification with the microarray data (Supplemental Table 5). We correlated 1057 proteins to probe sets from the microarray analysis. The reason a complete comparison was not made is because of the inability of the NetAffx (Affymetrix) software to match certain protein accession numbers to their associated microarray probe sets with high levels of certainty. We therefore concentrated analysis on data where co-identity of protein and probe set data was assured. Based on an analysis of the matched dataset we found a low correlation between the protein and microarray changes seen for each gene product (Table I). That is, many changes in protein levels could not be ascribed to altered mRNA expression levels. The lack of correlation spanned each pairwise comparison of the three sorted populations. The Bry +/- GFP Flk1- cells compared with GFP +/- Flk1- cells showed 120 cases where the protein was significantly increased yet mRNA level was not significantly changed; hence we have identified a substantial number of events that would not have been observed with the use of microarray analysis alone. This trend continued when Bry +/- GFP Flk1+ cells were compared with GFP +/- Flk1- cells. One hundred and seventy-nine significant protein changes (of a total of 188 protein changes) were identified where the mRNA expression was not altered. When the changes in expression level of the RNA and protein were considered in the Gly/GFP Flk1- and GFP +/- Flk1- Bry +/- GFP nine of the 188 protein changes correlated to changes in mRNA (Table I), whereas in GFP +/- Flk1- and GFP +/- Flk1- cells, 12 of 148 protein changes were seen at the RNA level as well.

Protein changes from Bry +/- GFP Flk1- cells and GFP +/- Flk1- cells primarily involved up-regulation (131 up-regulated and 17 down-regulated proteins). Of these 131 proteins, 48 are subsequently down-regulated as the cells differentiate toward the hemangioblast (GFP +/- Flk1- cells and GFP +/- Flk1- cells). This transitory expression helps to define the mesodermal development of ES cells.

A pairwise comparison of the protein and mRNA ratios between the populations was constructed to determine whether there was any trend in mRNA to protein changes between the two populations (Fig. 2, A and B). Previous studies have shown that in protein and RNA datasets the majority of genes are not altered in their expression level (41, 42, 54). Thus the data in Fig. 2 and Table I demonstrate that the vast majority of proteins and mRNA identified in this analysis are not altered. Bland-Altman plots were generated to assess the agreement between the protein and RNA ex-

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**Table I**

| mRNA up | mRNA no change | mRNA down |
|---------|----------------|-----------|
| GFP +/- Flk1-:GFP +/- Flk1- | | |
| Protein up | 8 | 120 | 3 |
| Protein unchanged | 8 | 883 | 18 |
| Protein down | 0 | 13 | 4 |
| GFP +/- Flk1-:GFP +/- Flk1+ | | |
| Protein up | 3 | 37 | 0 |
| Protein unchanged | 4 | 854 | 11 |
| Protein down | 0 | 142 | 6 |

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the associated change at the protein level in the nucleus (Fig. 2 and Tables I and II). There is neither a quantitative nor a qualitative relationship between changes in protein and mRNA levels for the majority of gene products (see also Supplemental Table 5).

To determine whether specific mRNA changes preceded protein changes (that is to say mRNA changes potentially required more time to influence the proteome) we also compared GFP\(^{+}\) Flk1\(^{-}\) :Bry\(^{+}\)/GFP ES cell proteome changes with GFP\(^{-}\) Flk1\(^{-}\) :GFP\(^{+}\) Flk1\(^{-}\) cell transcriptome changes. However, there was only a 5.35% correlation, which infers that there is little evidence for this argument as the association between protein and mRNA changes is as low as in previously described comparisons (Table I). Once again the majority of changes appear to be at the level of translation where many proteins show elevated and diminished expres-
sion with little alteration seen in the mRNA. Taken together these data indicate that there is no strong correlation between expression of nuclear proteins and transcription. Of course, inherent in the process of subcellular fractionation is the fact that some protein changes could be due to redistribution within the cell. Reference to the most complete study on nuclear proteins to date confirms a nuclear localization for most of the proteins in our dataset (49), but subcellular compartmentalization may affect correlation between nuclear protein and mRNA changes. Nonetheless there is a requirement to perform such subcellular fractionation to analyze changes in low abundance proteins (36, 55–59).

**Analysis of Gene Ontology on Gene Products Affected at the Protein or mRNA Level**—To determine whether (within gene products affected at the protein or mRNA level) there was an enrichment of genes involved in cellular processes that could infer a biologically relevant regulation of ES cell differentiation, protein and RNA data that were identified as changing were categorized by their gene ontology (GO). Fig. 3, A and B, shows the result of this analysis looking at the two developmental comparisons GFP $\textit{Flk1}^+$::GFP $\textit{Flk1}^-$ cells and GFP $\textit{Flk1}^+$::GFP $\textit{Flk1}^-$ cells. The protein and mRNA changes observed are spread between all the major gene ontology categories. Interestingly we see little change at the protein or RNA level in cell proliferation and differentiation gene products; however, there were large protein changes in DNA metabolism and protein biosynthesis. There is more apparent change seen at the RNA than the protein level in the developmental processes, and this includes chromatin packaging and remodeling with proteins such as high mobility group proteins.

A SOM was used to facilitate the identification of groups of proteins with similar expression patterns. A SOM is an unsupervised neural network algorithm that allows for the analysis of large datasets, uncovering patterns in the data and allowing for data visualization by way of a topological map. A SOM begins with random weight vectors, and the learning process is carried out by the modification of these weight vectors toward the data profiles to be mapped. Once training is complete the data are grouped so that different panels in the SOM represent cases whose profiles are similar. Various SOM size matrices were tested, and given that using larger size matrices were tested, and given that using larger size matrices resulted in empty clusters, a $1 \times 3$ map was the most appropriate for the generation of distinct cluster groups. The three cluster groups discovered by the SOM algorithm are summarized in Fig. 3, C, D, and E. Fig. 3F shows the median centroid for each cluster. Each cluster group has a unique expression pattern with cluster 1 showing an initial increase at the mesoderm stage and then a decrease to hemangioblast. Cluster 2 shows an increase to mesoderm and then an even sharper increase in expression to hemangioblast. Conversely cluster 3 shows the exact opposite with an overall decrease in the proteins belonging to this cluster. Statistical testing by the non-parametric Mann-Whitney $U$ test showed the differences in protein expression to be highly statistically significant, $p \leq 10^{-5}$, between all cluster groups at both the mesoderm and hemangioblast stages. Plotting a scatter plot to show the expression of the individual proteins at the mesoderm and hemangioblast stages also showed clear separation of the three cluster groups identified by the SOM (Fig. 3G).

Gene ontology information (Fig. 3, H, I, and J) revealed that the most striking changes are in cluster 1, which contains 16% of proteins with a role in protein biosynthesis, whereas no proteins have this role in cluster 2, and only 3% of proteins have this role in cluster 3. Cluster 2 has a large proportion of proteins involved in transport (9%) and intracellular protein trafficking (15%); however, these proteins are present at much lower percentages in clusters 1 and 2. The majority of protein changes for chromatin packaging and remodeling (6% of protein changes) are grouped into cluster 2. Immunity and defense is also well characterized in cluster 3 (13%) as compared with the other two clusters. It is also of interest that 5% of the proteins are involved in DNA metabolism, and all are associated with cluster 3. This computational approach has identified distinct clusters of proteins from expression profiling data that enable the identification of proteins sharing similar characteristics and also show a possible relation with biological process.

**Pathway Analysis of Proteins Changing in Expression Levels during Differentiation**—Proteins identified with a pivotal role in biological processes can be identified by a consideration of the connection between gene products using pathway analysis. Proteins associated to their respective RNA probe set showing altered expression in differentiating ES cells were therefore systematically assessed. This allowed identification of connections between the proteins that have altered expression levels in differentiating Bry $\textit{Flk1}^+$::GFP ES cells (Fig. 4, A–C). In the three comparisons made, GFP $\textit{Flk1}^+$::GFP $\textit{Flk1}^-$, GFP $\textit{Flk1}^+$.GFP $\textit{Flk1}^-$, and GFP $\textit{Flk1}^+$.GFP $\textit{Flk1}^-$, a number of proteins appear to be involved in the regulation of several gene products undergoing altered expression. RBL2 (also known as p130) has a central role in gene regulation. RBL2 is a member of the retinoblastoma (Rb) family of proteins and has been identified previously as being important in stem cell differentiation in several systems (60–62). RBL2 has been shown to have an antiproliferative activity involving the histone deacetylase (HDAC) pathway (61). RBL2 and HDAC1 have been shown to function together to inhibit transcription of the E2F-dependent promoter of cyclin A (63). E2F1 has also been included in the pathway analysis and is demonstrated to be associated with proteins whose expression level increases as the cells differentiate from GFP $\textit{Flk1}^+$ to GFP $\textit{Flk1}^-$. E2F1 is a transcription factor involved in the regulation of cell cycle mediators, e.g. cyclin E (64). High mobility group protein A2 (HMGA2) has been demonstrated to be involved in the regulation of Rb protein phosphorylation (65–68). In our study HMGA2 increases at the mRNA and protein level (see Fig. 5).
Fig. 3. Gene ontology of proteins whose levels change as Bry*GFP/+ ES cells differentiate. The gene ontology of all the proteins showing significant changes in expression levels between the three populations was analyzed by placing the proteins into 25 GO categories. A and B show a comparison of two Bry*GFP/+ ES cell populations at specific developmental stages. The mRNA changes (dark gray histograms) show the percentage of gene products within the dataset exhibiting a change. Values for each GO group were calculated as a percentage of total mRNA changes seen. The percentages of protein changes seen for each GO category are shown as light gray histograms. The changes are calculated with relation to total protein changes. Using a $1 \times 3$ map SOM size matrix proteins whose levels change were clustered into three groups (C, D, and E). F shows the median centroid for each cluster. G, a scatter plot to show the expression of individual proteins at the mesoderm and hemangioblast stages. H, I, and J, gene ontology information from the three clusters.
This analysis can therefore be used to identify novel proteins and pathways that could be involved in regulating stem cell differentiation. Other proteins that seem to be involved in the regulation of a number of genes include TNFRSF1A, also known as tumor necrosis factor receptor superfamily, member 1A. Tumor necrosis factor alpha has been shown to inhibit hematopoietic stem cell expansion and renewal (69).

Confirmation of Isobaric Tag/Tandem Mass Spectrometry Data on Protein Expression—To confirm the value of the iTRAQ approach for relative quantification in differentiating BrYellowGFP/H11001 ES cells we performed Western blot analysis on a number of proteins and coupled this to the real time PCR analysis and microarray data (Fig. 5). Previously UTF1, Sox2, and Oct4 have been demonstrated to be involved in maintenance of pluripotency in ES cells (13–16, 70). As confirmation of our protein and mRNA dataset the kinetics of Oct4, Sox2, and UTF1 were analyzed. It can be seen in Fig. 5A as the cells differentiate toward GFP/H11001ES cells these gene products are down-regulated at the mRNA and protein level as anticipated. The Western blot and real time PCR data also supported the microarray and the iTRAQ data, respectively, on nuclear proteins involved in chromatin packaging and remodelling or DNA metabolism such as HDAC2, HMGA2, and DNA methyltransferase 1 (DNMT1) (Fig. 5B). The Western blots and real time PCR experiments performed here confirm the data on these proteins and RNAs obtained from the microarray and iTRAQ method. The iTRAQ method for relative quantification of 2389 nuclear proteins was successful with the material required to run just a few Western blots. In Fig. 2, A–C, and Table I we demonstrated that there was limited correlation between protein and mRNA expression. We identified a number of differences at the mRNA level that did not change at the protein level. An example of this is for DNMT1: the Western blot data and the iTRAQ data were in agreement but did not reflect the situation seen with real time PCR analysis of mRNA levels or microarray data. Systematic proteomics analysis correlated to Western blot analysis in the proteins highlighted here (see Fig. 5). Thus the isobaric tag data obtained can be compared with microarray data (see Fig. 5), which in turn are validated by real time RT-PCR data. This represents a high penetrance approach to understanding stem cell development.

DISCUSSION

The process of embryonic cell development to mesoderm-committed cells and hemangioblast can be studied using the BrYellowGFP/H11545 ES cells (28). As they offer sufficient biological material for systematic analysis, we have taken advantage of the possibility for enriching cells at a specific stage of development to undertake mRNA and proteomics analyses. The latter analysis was considered essential as there is evidence of a decrease in expression levels. Yellow represents proteins that were not found in our proteomics analysis but have previously been associated with proteins that were identified.
disconnection between mRNA and protein expression levels for a variety of gene products in enriched hematopoietic stem cells as they undergo development (41). Here we confirm that this observation extends to BryGFP/H11001 ES cells differentiating toward a mesodermal lineage cell fate. Although proteomics studies have been performed on ES cells using two-dimensional gel electrophoresis (71–73) we have developed a novel form of analysis with increased penetration and quantitative accuracy (42) that is now applied to ES cells. The benefits of using a quantitative multidimensional LC-MS/MS approach include a greater ability to analyze hydrophobic, large, and basic proteins, the latter of importance with respect to nuclear proteins such as histones. Furthermore in our hands multidimensional LC-MS/MS incorporating iTRAQ methodologies offer greater proteomic penetration than two-dimensional gel techniques (74, 75).

Our data show that we have identified and relatively quantified 2389 proteins of differentiating BryGFP/+ ES cells. This is one of the largest relatively quantified mammalian proteomics datasets published to date. The preparation of nuclei for proteomics analysis was a requirement to identify changes in transcriptional mechanisms associated with differentiation. It is also widely accepted that prefractonation is required for systematic analysis of low abundance proteins (57–59). Proof of this principle is demonstrated in this report with the identification and relative quantification of low abundance transcription factors, e.g., Sox2, Oct4, UTF1, and E2F4. The majority of proteins quantified have been shown to reside (at least in part) in the nucleus (49). This includes proteins such as actin and cofilin, which have been regarded as non-nuclear proteins until recently (76, 77).

Having enriched ES cell populations at specific stages of development and compared proteomic changes to microarray analyses we observe that there is poor statistical agreement between the two. This is supported by previous studies (36–40). Interestingly Unwin et al. (41) described an mRNA and proteome comparison of murine hematopoietic stem cells and demonstrated a lack of correlation in 54.7% of the identified genes. In our study the comparison of GFP Flik1− cells with GFP+Flik1− cells demonstrated that 89.9% of the pro-

FIG. 5. A comparison of relative expression levels of proteins and mRNA validated with the use of real time PCR and Western blot. A, Oct4, UTF1, and Sox2, known regulators of pluripotency, were identified in our proteomics and microarray analysis and validated with the use of Western blot and real time PCR, respectively. B, validation of the protein and mRNA expression levels of HMGA2, HDAC2, and DNMT1. Western blots were performed with both nuclear and whole cell preparations. The iTRAQ data for each protein is shown (white histogram) with data from real time PCR analysis (line) and microarray (gray histogram). Error bars shown are from three observations and represent the S.E.
teins that showed a significant change were shown as no change in the microarray analysis. The GFP “Flk1” cells compared with the GFP “Flk1-” Bry+/GFP cells also demonstrated a pattern of more protein changes than RNA changes (188 compared with 24), and more interesting still 95% of protein changes were not associated with mRNA changes (see Table I). A statistical agreement between protein and mRNA changes would indicate regulation of these genes at the level of chromatin remodeling and transcription (78). However, as discussed, the majority of the significant changes seen at the mRNA or protein levels do not agree. Regulation of these genes must be at the level of translation and/or the relative degradation rates of the mRNA and protein. Alternatively there is a possibility that our results are explained by a temporal lag between changes in mRNA levels and changes in protein levels. We have addressed this issue by comparing early mRNA changes in GFP “Flk1-” ES cells with later stage changes in the proteome (GFP “Flk1-”::GFP “Flk1”), yet there are still few correlations between mRNA and protein modulations. Where small incremental changes in development are being compared there will be low correlation between protein and mRNA changes. Stem cells have been shown to produce mRNA for a large number of genes, and this decreases with differentiation (79). One hypothesis is that the mRNA species generated in stem cells are not directly linked to production of protein. They are produced to offer multiple options for development. An extension of this is that as cells develop increased and decreased expression of mRNA species offers differential roads to develop. This suggestion supports the need for systematic analysis at the protein level as well as a microarray-based experiment. Nonetheless the extensive mRNA analysis of ES cells to date has of course yielded data on pathways for self-renewal and differentiation (80, 81). Networks of transcription factor expression and transcription factor protein interaction can be seen to govern differentiation. Our data indicate an increased level of complexity to this system; whereas transcription factor complexes bind to promoter regions to initiate mRNA production, the direct relation between these critical events and their downstream effects are now less clear. Modulation of transcription does not directly govern the levels of many nuclear proteins. This offers a further layer of complexity to the governance of normal differentiation processes. The biological and analytical impact of this piece of work is that it demonstrates that changes at the RNA level cannot be used to assume protein expression levels. Recently Doss et al. (82) have shown that an embryonic stem cell line that was differentiated and analyzed using microarray demonstrated 479 genes up-regulated and 193 genes down-regulated. However, due to the lack of agreement seen between protein and RNA expression levels in differentiating stem cells these changes may not be seen at the protein level. Post-transcriptional regulation by micro-RNA is one mechanism that can explain the data reported here (83). Another is the compartmentalization of (well defined) nuclear proteins to the cytosol, an unlikely scenario for so many proteins.

We confirmed selected iTRAQ-derived information on modulation of protein levels using Western blot analysis. This gives a high level of confidence that isobaric tag-based mass spectrometry can permit the sensitive relative quantification data acquisition that permits the above conclusions. DNMT1 associates with HDAC proteins and cooperates with retinoblastoma protein to repress transcription from promoters containing E2F-binding sites (84–86). DNA methylation and histone deacetylation are key in the regulation of transcription, yet these two proteins show invariant expression in early ES cell development (Fig. 5). In the case of DNMT1, however, the mRNA level is shown to decrease during differentiation (see Fig. 5). UTF1 is a transcriptional co-activator that has been found to be expressed mainly in pluripotent ES cells (87). The regulatory elements governing UTF1 gene expression include octamer binding transcription factor Oct4. Our data clearly show that Oct4 protein expression decreases as UTF1 expression decreases (Fig. 5). Perhaps surprising is the fact that the Oct4 protein only decreases to a degree before UTF1 becomes undetectable by Western blot. These are examples of the opportunities arising from isobaric tag datasets for network building on gene expression/protein regulation.

We developed an interaction network of proteins identified as changing in expression during ES cell development (Fig. 4). We identified HMGA2 as a protein changing in expression. This protein has a linkage to E2F1, E2F4, and SP1 as well as to HDAC1 and HMGB2. HMG proteins are transcription factors that are expressed during embryonic development (88, 89) and are involved in a network of protein-protein and protein-DNA interactions resulting in the formation of enhancers at promoters (90, 91). In vitro studies have demonstrated that the HMGA2 protein was able to efficiently bend DNA and form DNA circles, a role of potential significance in V(D)J recombination (92). These studies suggest a role in facilitating cooperative interactions between cis-acting proteins by promoting DNA flexibility. The HMGA2 gene is commonly overexpressed in neoplastic cells and is frequently amplified in adenomas, apparently affecting tumor formation via the E2F1 protein (93). Knock-out mouse studies of the HMGA2 gene led to a pygmy phenotype (89). Bmi1 is a target of E2F1 and has a pivotal role in embryonic development and self-renewal in embryonic and hematopoietic stem cells (94, 95). Thus the proteomics data we have acquired offers opportunities to build hypotheses on processes for self-renewal regulation in primitive cells. The HMGB2 protein (identified by iTRAQ and microarray; no change in expression levels) is known to interact with HMGA2 and Oct4 (96). The related HMGB3 protein (seen to increase when the cells differentiate from epiblast to mesoderm at the protein and mRNA level by iTRAQ and microarray) regulates the generation of myeloid and lymphoid progenitor cells in the hematopoietic system. It also regulates the self-renewal of hematopoietic stem cells.
(97, 98). These data exemplify the opportunities that deep proteomics mining of ES cells undergoing differentiation offers to systematically analyzing gene product function in development.

It can be argued that the use of the system we have described is a rapid screening system to precede more focused hypothesis-driven research. Thus the development of techniques to study the translational regulation of protein levels decreases the reliance on proteomics confirmation of mRNA-based analyses and offers opportunities to develop new quantitative biology approaches to the understanding of stem cell self-renewal or differentiation with a greater probability of discovering proteins that regulate development. Protein interactions can similarly be mapped in ES cells for systematic discovery of regulatory pathways (99). Here we have presented validation of a discovery proteomics approach to stem cell biology.

* This work was supported by the Biotechnology and Biological Sciences Research Council, Leukemia Research Fund, and Cancer Research UK. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[5] The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

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