Dynamic Proteomics Reveals High Plasticity of Cellular Proteome: Growth-Related and Drug-Induced Changes in Cancer Cells are Comparable

In chemical proteomics, the changes occurring in cellular proteomes upon drug treatment are used to identify the drug targets and the mechanism of action. However, proteomes of cultured cells undergo also natural alteration associated with changes in the media, attaining a degree of confluence as well as due to cell division and cell metabolism. These changes are implicitly assumed to be smaller in magnitude than the drug-induced changes that ultimately lead to cell demise. In this study, it is shown that growth-related proteome changes in the untreated control group are comparable in magnitude to drug-induced changes over the course of 48 h treatment. In two well-characterized cancer cell lines, growth-related effects assessed with deep proteomics analysis (10 481 proteins quantified with at least two peptides) show common trends, the steady downregulation of cell division processes, and the upregulation of metabolism-related pathways. The magnitude of these variations, which are present even before reaching 100% confluence reveals unexpectedly high plasticity of the cellular proteome. This finding reinforces the need, generally accepted in theory but not always followed in practice, to use a time-matched control when measuring drug-induced proteome changes.

1. Introduction

With the recent advances in mass spectrometry (MS), tandem MS (MS/MS), and liquid chromatography (LC) instrumentation, expression proteomics has become a tool of choice to study comparative variations of protein abundances across the whole proteome upon any perturbation.[1] As a routine, a single LC-MS/MS experiment produces quantitative information on ≥5000 proteins[2–5] which encompasses a significant part, perhaps a majority, of the meaningfully expressed cellular proteome (i.e., the proteome that materially affects cell state). Great improvements have also been made in the accuracy of the quantitative analysis, with a reliably achieved median coefficient of variability (CV) of protein abundances between the technical and even biological replicates reaching below 10%. These improvements enabled a multitude of studies that are critically dependent upon accurate measurements of the changes in protein expression levels. As an example, Functional Identification of Protein Targets by Expression Proteomics[7–10] uses changes in cellular proteomes upon long-term (48–72 h) exposure to anticancer drugs to identify their protein targets as well as the mechanism of action. Such drug target deconvolution approaches are currently of particular interest, as an increased number of drugs are coming to clinical trials with poorly understood targets and molecular action.[11,12] Moreover, the drugs discovered via target-blind phenotypic screenings currently represent a significant part, in some periods even a majority, among the novel FDA approved compounds.[13,14] Since unbiased uncovering of targets and mechanisms of action of small-molecule drugs represents one of the most important application areas of modern proteomics,[15–17] accurate assessment of proteome changes is of foremost importance.

The generally known but often neglected fact that has significant implications for the above efforts is the dynamic nature of the cellular proteome even at the conditions of unperturbed growth. Indeed, as the cells grow in culture, the concentration of nutrients and excreted molecules, the pH of the media, and the attained degree of cell confluence vary significantly. Additional factors affecting proteome changes are the cell division, metabolic, redox, and circadian cycles.[18] These changes in the average cellular proteome are often assumed to be smaller in magnitude than the drug-induced changes that ultimately lead to cell demise, and sometimes they are even deemed irrelevant. It is not unusual to find in literature studies where the cells treated...
with a drug for several hours are compared with untreated control at time zero. The implicit assumption that cell death pathways affect the cellular proteome more significantly than natural processes during normal cellular growth has however never been tested experimentally.

There are reasons to suspect that the proteome changes under natural growth may be quite significant. There is a body of research that demonstrates the cell proteome to be a highly dynamic system. One source of the variation in specific protein’s expression is the circadian cycle in mammalian cells. Recently, Alon et al. studied the level and localization of pathways. 

They found that 40% of the proteins exhibited cell cycle dependence, of which 11% showed changes in abundance and 35% in localization. A comparable fraction of the measured proteome was found changed in our studies of drug-related alterations after 24–72 h.

Thus in this study, we aimed to compare the proteome changes in natural growth with those occurring under the influence of drugs. For the pilot experiment, we chose HCT116 colon cancer cells treated by methotrexate (MTX), paclitaxel (PCTL), or vehicle (equivalent to untreated control). The drug concentrations corresponded to 50% reductions in cell counts after 48 h compared to vehicle treatment. Such a system has been found to be well-behaving in our earlier studies that is, the known drug targets showed more significant regulation than other proteins. To assess the dynamics of proteome changes, we first collected the cells and analyzed them every 6 h for 48 h of growth. The proteomics data were mapped using principal component analysis (PCA), and the shifts on the PCA scale was taken as a quantitative measure of the proteome change. The initial expectations were that the time variations in untreated or vehicle-treated cells will be relatively small compared to drug-induced changes. These expectations were, however, shattered by the experimental results that showed equivalent variations in both cases. This induced us to analyze untreated cancer cells in more detail, and to produce an in-depth time series of untreated A375 and HCT16 grown for 48 h. This analysis revealed time-dependent changes in expression levels of proteins related to cell division and metabolism pathways.

2. Experimental Section

2.1. Reagents and Chemicals

All reagents and chemicals were purchased from Sigma-Aldrich unless otherwise specified.

2.2. Cell Culture

Human colon colorectal carcinoma HCT116 and human malignant melanoma A375 cells obtained from ATCC were grown in DMEM (Lonza, Wakersville, MD, USA) supplemented with 10% FBS superior (Biochrom, Berlin, Germany), 2 mM l-glutamine (Lonza, Wakersville, MD, USA), and 100 units per milliliter penicillin/streptomycin (Gibco, Invitrogen), at 37 °C with 5% CO₂.

2.3. Cell Treatments

CellTiter-Blue Cell Viability Assay (Promega) was used to determine the half maximal inhibitory concentration (IC50) values for MTX and PCTL according to the manufacturer’s protocol. Briefly, cells were cultured in 96-well plates at a density of 4000 cells per well (100 μL total volume per well) and after attachment were treated for 24 h with serial concentrations of MTX and PCTL. After 48 h, 20 μL of resazurin were added to each well. Subsequently, plates were incubated at 37 °C for 2 h. The plates were shaken for 10 s and fluorescence was recorded at 560/590 nm using Epoch microplate spectrophotometer (BioTek). For proteomic analyses of treated cells, HCT116 was seeded into 6-well plates at a density of 200 000 cells per well. Twenty-four hours later, cells’ medium was replaced with fresh medium containing 25 nm of PCTL, 45 nm of MTX, or 4.5 ppm of DMSO (vehicle) corresponding to the maximum concentration used in the experiment. The cultures were then further incubated and two replicate samples were collected every 6 h for each treatment and control. Samples corresponding to cells without any treatment (time 0 [T0]) were also collected in the beginning of the experiment (Figure 1).

Before collection, cells were washed twice with PBS and subsequently, lysis buffer (3% sodium deoxycholate [SDC] and 50 mM ammonium bicarbonate) was added to each well. Cells were then collected into microtubes, heated at 80 °C for 10 min before being sonicated using a Branson probe sonicator (3 s on, 3 s off pulses, 45 s, 30% amplitude). Protein concentration was measured using Pierce bicinchoninic acid assay (BCA) protein assay kit (Thermo) according to the manufacturer’s protocol. Subsequently, 100 μg of proteins were reduced by adding dithiothreitol to a final concentration of 15 mM and incubated at 60 °C for 30 min, followed by alkylation using a final concentration of 20 mM iodoacetamide for 45 min in the dark. SDC concentration was reduced to 1.5% and lysyl endopeptidase (LysC)

Significance Statement

Naturally occurring proteome changes over time in in vitro cell cultures are well known in principle but often overlooked in practice as they are usually assumed to be insignificant compared to drug- or siRNA-induced changes. Thus the rule that time series measurements should be compared with time-matched controls is sometimes disregarded in practice. Incidentally, we found the natural variations in cellular proteome to be surprisingly large, and decided to study them in detail. Here, we found using a time series proteomics analysis that the changes in controls cells (vehicle-treated or untreated) are on par with the proteome changes induced by drug treatments. Additionally, we produced for A375 and HCT116 cancer cells lines a time series deep proteomics dataset encompassing 10 481 proteins that could be used as future reference for in vitro cell growth effects. The surprising observations of great plasticity of the cellular proteome strengthen the need for time-matched controls in proteomics experiments and more generally for careful considerations of in vitro cell culture–based assay readouts.
TMT10plex-labeled, which was followed by deep proteomics analysis. Maximum integration time of 50 ms. The acquire data resolution of 120000 with a target value of 1.10^5 or from the most abundant ion (top-20 method). MS spectra were acquired from the instrument, fragmenting up to 20 peptides per cycle starting between MS and MS/MS modes using the maximum speed of the instrument. Fragmenting up to 20 peptides per cycle starting between MS and MS/MS modes using the maximum speed of the instrument in a data dependent manner with automatic switching according to approximately 1 μL.

Proteomics workflow. HCT116 cells (n = 2) were treated with vehicle, MTX, and PCTL and incubated for 48 h, with a sample taken every 6 h and analyzed by label-free proteomics. Also, HCT116 (n = 3) and A375 cells (n = 3) were grown for 48 h; samples were taken every 12 h, digested and TMT10plex-labeled, which was followed by deep proteomics analysis.

(Merck KGaA) was added at a 1:100 ratio (LysC/protein, w/w), and the samples were incubated at room temperature overnight. SDC concentration was reduced further to 0.5% and modified sequencing grade trypsin (Promega) was added at a 1:100 ratio (trypsin/protein, w/w) and incubated for 6 h. Acetic acid (5% v/v) was then added to precipitate SDC and quench the reaction by incubating at room temperature for 30 min. The samples were centrifuged at 20 000 × g for 15 min and the supernatant was collected. Finally, the samples were desalted using StageTip (Thermo) according to manufacturer’s protocol, dried using SpeedVac, and resuspended into 0.1% formic acid (FA) (Fluka) prior to LC-MS/MS analysis.

2.4. LC-MS/MS Analysis

Peptides were separated on a 50 cm EASY-Spray column, with a 75 μm internal diameter, packed with 2 μm PepMap C18 beads, having 100 A pores (Thermo Fischer Scientific). An Easy-nLC-1000 system (Thermo Fischer Scientific) was used programmed to a 2 h optimized11 LC gradient. The two mobile phases consisted of buffer A (98% Milli-Q water, 2% ACN, and 0.1% FA) and buffer B (98% ACN, 2% Milli-Q water, and 0.1% FA). The gradient started with 2% B for 1 min, increased to 5% B in 7 min, 17% B in 65 min, 30% B in 20 min, and then to 95% B in 10 min, stayed at 95% B for 5 min and finally decreased in 2 min to 2% B that stayed for 10 min. The injection was set to 5 μL corresponding to approximately 1 μg of peptides and the samples were analyzed in a random order.

Mass spectra were acquired on an Orbitrap Fusion mass spectrometer in a data dependent manner with automatic switching between MS and MS/MS modes using the maximum speed of the instrument, fragmenting up to 20 peptides per cycle starting from the most abundant ion (top-20 method). MS spectra were acquired at a resolution of 120 000 with a target value of 1.10^5 or maximum integration time of 50 ms. The m/z range was from 350 to 1500. Peptide fragmentation was performed using higher-energy collision dissociation (HCD), and the energy was set at 28%. The MS/MS spectra were acquired at a resolution of 30 000 with the target value of 5.10^4 ions and a maximum integration time of 50 ms. The isolation window and first fixed mass were set at 1.6 m/z units and m/z 120, respectively.

2.5. Deep Proteome Analysis of Untreated Cells

HCT116 and A375 cells were seeded at 150 000 cells per well in 6-well plates in triplicates 24 h prior to the start of the experiment and then cells were collected at time 0 (start of the experiment), 12, 24, 36, and 48 h into a media containing 1% SDS, 8 μL urea, and 50 mM Tris and sonicated on ice using a Branson Probe Sonicator (3 s on, 3 s off pulses, 45 s, 30% amplitude). Pictures of the cells at each time point were recorded using a Primo Vert microscope equipped with an AxioCam ERC 5s camera (Zeiss) with 4× magnification. Protein concentration was measured using the BCA assay; 50 μg of protein from each sample was reduced using 5 mM DTT at RT for 1 h followed by alkylation using 15 mM IAA in the dark at RT for 1 h. The reaction was quenched with 10 mM DTT and the samples were precipitated using methanol chloroform. The dried protein pellets were resuspended into 8 μL urea, 20 mM EPPS pH 8.5, the urea concentration was lowered to 4 M by adding 20 mM EPPS pH 8.5 and LysC digestion was conducted at a 1:100 ratio (LysC/protein, w/w) overnight at RT. Then, the urea concentration was reduced to 1 M and trypsin digestion was conducted at a 1:100 ratio (trypsin/protein, w/w) at RT for 6 h. TMT10plex (Thermo Fischer Scientific) reagents were resuspended into dry ACN at a concentration of 20 μg μL−1 and 200 μg were added to each sample (four times the peptide quantity according to manufacturer’s protocol). The ACN concentration in the samples was adjusted to 20% and the labeling was conducted at RT for 2 h and quenched with 0.5% hydroxylamine (Thermo Fischer Scientific) for 15 min at RT. The samples were then
combined and dried using SpeedVac to eliminate the ACN. Then, the samples were acified to pH < 3 using TFA and desalted using SepPack (Waters). Finally, samples were resuspended into 20 mM NH₄OH and 150 μg of each sample was used for off-line fractionation.

Samples were off-line high-pH reversed-phase fractionated using an Ultimate 3000 RSLCnano System (Dionex) equipped with a XBridge Peptide BEH 25 cm column of 2.1 mm internal diameter, packed with 3.5 μm C18 beads having 300 Å pores (Waters). The mobile phase consisted of buffer A (20 mM NH₄OH) and buffer B (100% ACN). The gradient started from 1% B to 23.5% B in 42 min, then to 54% B in 9 min, 63% B in 2 min and stayed at 63% B for 5 min and finally back to 1% B and stayed at 1% B for 7 min. This resulted in 96 fractions that were concatenated into 24 fractions. Samples were then dried using SpeedVac and resuspended into 2% ACN and 0.1% FA prior to LC-MS/MS analysis.

Peptides were separated on a 50 cm EASY-Spray column, with a 75 μm internal diameter, packed with 2 μm PepMap C18 beads, having 100 Å pores (Thermo Fischer Scientific). An Easy-nLC-1000 system (Thermo Fischer Scientific) was used programmed to a 90 min optimized LC gradient. The two mobile phases consisted of buffer A (98% Milli-Q water, 2% ACN, and 0.1% FA) and buffer B (98% ACN, 2% Milli-Q water, and 0.1% FA). The gradient started with 5% B and increased to 10% B in 5 min, 26% B in 90 min, 90% B in 5 min, and finally stayed at 90% B for 10 min. The injection was set to 5 μL corresponding to approximately 1 μg of peptides.

Mass spectra were acquired on a Fusion Orbitrap mass spectrometer (Thermo Fischer Scientific). The Fusion acquisition was performed in a data dependent manner with automatic switching between MS and MS/MS modes using a top-20 method. MS spectra were acquired at a resolution of 120 000 with a target value of 1.10⁶ or maximum integration time of 50 ms. The m/z range was from 400 to 1600. Peptide fragmentation was performed using HCD, and the energy was set at 40%. The MS/MS spectra were acquired at a resolution of 60 000 with the target value of 1.10⁵ ions and a maximum integration time of 105 ms. The isolation window and first fixed mass were set at 1.4 m/z units and m/z 105, respectively.

2.6. Protein Quantification

Protein identification and quantification were performed using MaxQuant software version 1.5.3.30 for the label-free dataset and version 1.6.3.3 for the TMT10plex-labeled samples. Acetylation of N-terminal, oxidation of methionine, and deamidation of asparagine and glutamine were selected as variable modifications. Carbamidomethylation of the cysteine was selected as fixed modification. The Andromeda search engine was using the International Protein Index (human version 2014,02, 89 054 entries) for label-free search and with Uniprot database (human version 2018,06, 93 614 entries) with the precursor mass tolerance for the first searches and the main search set to 20 and 4.5 ppm, respectively. Trypsin was selected as the enzyme, with up to two missed cleavages allowed; the peptide minimal length was set to seven amino acids. Default parameters were used for the instrument setting. The FDR was set to 0.01 for peptides and proteins. LFQ was chosen as a quantification parameter with a minimum ratio count of 2. “Match between runs” option was used with a time window of 0.7 min and an alignment time window of 20 min. Only proteins with at least two peptides were considered.

2.7. Data Processing and Statistical Analyses

Analysis of protein abundances was performed in R (version 3.5.0). Protein abundances were normalized by the sum of the total abundance of all proteins in the corresponding sample. Ratios with sample T0 were then calculated for all proteins and samples. Any protein with missing values was excluded from the statistical analysis. p-value calculations were performed using a tailed paired t-test. SIMCA (Version 14, UMetrics, Sweden) was used for PCA.

For the deep proteome experiment, the coefficient of variation (CV) was calculated for each protein between the replicates. Only proteins having an average CV <15% were kept for the analysis, which resulted in 6053 proteins. Heatmaps were created using manhattan and ward.D2 as clustering methods. Pathway analysis was performed using gene ontology (GO) annotations, with p-values adjusted using Benjamini–Hochberg correction.

3. Results and Discussion

3.1. Vehicle-Treated Proteome Changes Greatly Over Time

In total, 4700 proteins were quantified with two or more unique peptides across the whole HCT116 dataset (Table S1, Supporting Information). Abundances of 2116 proteins quantified in all samples were submitted to PCA (Table S2, Supporting Information). The plot of first two components (Figure 2A) shows gradual separation over time of all samples and controls from T0, mostly along the 1st principal coordinate t1. Remarkably, the vehicle-treated proteome moved after 48 h along t1 more than the PCTL treatment, and as much as the MTX treatment after 30 h. This effect, which is likely due to the changing environmental factors in culture media, such as the availability of nutrients and growth factors, acidity, degree of confluence, etc. has previously been known qualitatively; however, its relative magnitude at the proteome level has, to the best of our knowledge, not been investigated. That unperturbed growth of cells can be associated with a similar magnitude change in the proteome as a toxic shock killing half of the cells is a nontrivial fact that surprised us despite the decade long experience in quantitative proteomics.

3.2. Vehicle-Treated Proteome Changes are Gradual and Global, on Par with Changes Induced by Paclitaxel Treatment

In order to evaluate whether the proteome changes are global (affected by many proteins) or only due to high regulation of a few specific proteins, the mean (among the replicates) of the abundance ratios with T0 and the corresponding p-value were calculated for 2116 common proteins. The number of proteins exhibiting a significant change in expression (p-value < 0.05) was
found to gradually increase for all conditions starting from 6 h, reaching maximum at 48 h (Figure 2B). Similar behavior is observed for the distribution of protein expression fold change between each time point and T0 (Figure 2C). The MTX treatment induced the highest variation compared to the two other conditions and the distribution is also more stretched. Vehicle-treated proteome behavior is on par with PCTL treatment for all the plots in Figure 2.

Thus, changes in the protein expression appear to be gradual and global, rather than being an exceptional behavior of a few specific proteins. This conclusion applies to both treatments and the vehicle-treated group. Note, however, that the changes are not monotonic: for example, for MTX the number of upregulated proteins at 42 h is smaller than at 36 h, and the number of downregulated proteins does not change significantly in the 24–36 h interval. A similar effect is observed for PCTL and vehicle-treatment.

### 3.3. The Proteome Deviation with Time is Not Due to Chemical Treatment and is Not Cell Type Specific

To confirm our findings regarding the vehicle-treated proteome, the experiment was repeated in triplicate with A375 and HCT116 cells in the absence of any chemical treatment and using 12 h time intervals up to 48 h; 10 481 proteins were quantified in total and 8898 proteins were quantified in all samples with at least two peptides (Table S3, Supporting Information). The violin plot using the 6053 proteins with average CV <15% shows a similar trend as what was observed in HCT116 cells treated with DMSO in the first experiment (Figure 3A; Table S4, Supporting Information). Indeed, the variation in the data gradually increases both for HCT116 and A375 until 48 h where the data is the most scattered. Surprisingly, photographs of the cells taken at each time point (Figure 3B) show that at 24 h, when the deviation of the proteome from T0 start to become large, for both cell lines the confluence is still ≤50%, and even at 48 h where the deviation is at its highest, the cells do not reach 100% confluence. Thus, growth and environmental effects start to have a noticeable impact on the cellular proteome much earlier than expected. Photographs also show that HCT116 is slightly less confluent than A375 at each time point which appear to correlate with smaller HCT116 proteome deviation from T0 (Figure 3A). Finally, the results demonstrate that even renewing the cell culture medium every day or every 2–3 days as it is widely accepted would not eliminate such proteome variations. Therefore, the proteome variations that we recorded in the first experiment are not due to chemical treatment and are not specific to the HCT116 cell line.

### 3.4. Cell Division and Metabolism-Related Pathways are the Most Affected during In Vitro Cell Culture

In order to investigate common features between A375 and HCT116 cells that would reflect general response to prolonged in vitro cell culture, all the quantified and filtered proteins were
clustered according to their fold change compared to T0 (Figure 4). The clusters 2 and 10–14 on the heat map encompass proteins showing gradual and similarly large changes in the same direction (up or downregulation) in both cell lines, while clusters 1, 5, and 7–8 correspond to proteins with cell-specific and more moderate changes. The heat map confirms the previous observation of higher variations in A375 cells than in HCT116 cells, probably due to the division rate difference that reflects in different degrees of confluence.

The GO enrichment analysis revealed three main downregulated processes in both cell lines, namely cell division pathways in cluster 14 as well as ribosome biogenesis and DNA replication–related pathways in cluster 2. The upregulated clusters 6 and 12 are most enriched with proteins participating in redox processes and respiration as well as organic acid metabolism. Clusters 10, 11, and 13 are among the most regulated but are not significantly enriched by any pathway. Cluster 11 is the most downregulated; it consists of 13 proteins, of which JUN, CTGF, and IGF2 are all involved in cell proliferation (Table S4, Supporting Information). The trend is a global downregulation of cell division processes with incubation time and an upregulation of metabolic pathways, including energy production. This is hardly surprising as cell confluence increases over time, which ultimately triggers cell contact inhibition and leads to an overall decrease of the cell division rate. On the other hand, nutrient levels in the media are decreasing over time, which the cells are likely trying to compensate by upregulating metabolic and energy production pathways.

Remarkably, the heat map demonstrate that changes in cell cycle and metabolism-related proteins expression are noticeable as early as 12 or 24 h after the start of the experiment with a relatively low cell confluence (40–60% after 24 hours) which is still considered to be the exponential growth phase. Therefore, our data show that the effect of in vitro cell culture on the cellular proteome begins to appear at a relatively low confluence.

4. Concluding Remarks

The most important finding in this work is that the cellular proteome has higher plasticity than previously assumed: the
vehicle-treated group showed proteome variations on a similar scale as the drug-treated group. These observations are in line with the common (and yet not always followed in practice) belief emphasizing the importance of same-time controls in all biological experiments. Therefore, large proteome variations in in vitro cell culture happen regardless of the culture conditions even in a tightly controlled environment.

Here, we provide a deep proteomics dataset for two untreated cancer cell lines freely growing for 48 h that can be used as a reference for monitoring proteome changes in in vitro cell culture as well as for studying uncertainties in global protein expression levels in these two cell lines. We also provided a quantitative estimate of the magnitude of observed proteome variations that are usually overlooked in terms of protein fold change. Finally, our analysis demonstrates that cell cycle–related protein expression changes arise at a relatively low confluence.

The mass spectrometry proteomics data have been deposited to ProteomeXchange Consortium (http://proteomexchange.org) via the PRIDE partner repository with data set identifiers PXD007762 and PXD010805.

Figure 4. Left—Heat map of mean protein fold changes at each time point compared to T0; right—Fold enrichment of GO processes, with bars colored according to the average fold change compared to T0 at 48 h for both cell lines combined.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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