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Bottom-up proteomic analysis of single HCT 116 colon carcinoma multicellular spheroids

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Abstract

Rationale—Proteomic analysis of single multicellular spheroids has not been previously reported. As three-dimensional cell cultures are an increasingly popular model system for biological research, there is interest in obtaining proteomic profiles of these samples. We investigated the proteome of single HCT 116 multicellular spheroids using protocols optimized for small sample sizes.

Methods—Six biological replicates were analyzed via microscopy for size. Total protein content was assessed via the bicinchoninic acid assay (BCA assay). Five separate biological replicate spheroids were analyzed via mass spectrometry in technical duplicate. An ultra-performance liquid chromatography (UPLC) system coupled with an LTQ Orbitrap Velos was used for peptide separation, analysis, and identification.

Results—The average diameter of six replicate HCT 116 spheroids was $940 \pm 30 \mu\text{m}$ and the average total protein amount was determined to be $39 \pm 4 \mu\text{g}$. At least 1300 protein groups were identified in each single LC-MS/MS run with ten percent of the material from single spheroid loaded. Database search results showed variation between spheroid protein group identifications. Pearson correlations show that the disparity in identifications is due to random variations in spectra and protocol.

Conclusions—We detected more than 1350 protein groups in each replicate HCT 116 spheroid. While some variation was detected between replicates, differences in the number of protein groups identified were determined to be the result of random variations in mass spectra acquisition.

Introduction

Three-dimensional (3D) cell cultures are valuable model systems for biological research and have a number of advantages as compared to other model systems. Similar to two dimensional (2D) monolayer or suspension tissue culture models, they are versatile and relatively rapid models of cellular growth. Changes in cell-cell communication, paracrine signals, and other pathways result in more accurate cellular microenvironments than cells grown directly on plastic. 3D cultures allow for the chemical microenvironment from the original organism to be recapitulated^{1,2}. These characteristics give 3D cultures excellent

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predictive value for traits such as protein expression and drug resistance³. Depending on the specific cell culture conditions, they can be cultured in high throughput configurations, with both 96- and 384-well plates as common culture supports. They are considerably less expensive than animal models.

Another substantial advantage of 3D cell cultures is their reproducibility. As we demonstrate in this report, 3D cell cultures prepared with clonal cell lines under the same culturing conditions are remarkably consistent in terms of their overall size and protein content. These cultures are usually analyzed in bulk, which provides ample material for analysis. However, bulk assays obliterate any information on the heterogeneity in composition between spheroids. The ability to study a single 3D cell culture enables the identification of patterns that may not be captured by the ensemble average. In this report, we demonstrate the application of microproteomic sampling and analysis strategies for the examination of individual 3D cell cultures. To our knowledge, this is the first mass spectrometric-based proteomic evaluation of single 3D cell cultures.

3D cell cultures have previously been analyzed with mass spectrometry. Several studies have focused on the differences between 2D monolayer and 3D cell culture. Gaedtke *et al.* analyzed low-passage colon carcinoma cells in 2D and 3D cultures⁴; similarly, Kumar *et al.* examined the differences between 2D and 3D neuroblastoma cell cultures⁵. Both found several differentially expressed protein groups, especially among structural and metabolic proteins. Paul *et al.* reviewed the discovery of cancer biomarkers with 3D cell culture and mass spectrometry-based proteomics, many of which are used clinically⁶. McMahon *et al.* characterized 3D cell culture layers following serial trypsinization of consecutive layers of cells from multiple cultures. Using an iTRAQ approach, they quantified abundance differences in lipid and steroid biosynthesis proteins in the proliferative outer cell layer, the quiescent intermediate cell layer, the perinecrotic region, and necrotic core cells⁷. Vliet *et al.* performed neurotoxicology studies using 3D cell culture models and mass spectrometry for metabolomics of possibly harmful chemicals in brain tissue⁸. Imaging mass spectrometry has seen many uses for 3D cell cultures for detection and evaluation of therapeutics^{9,10} and for proteomic studies^{11,12}. While all of these previous investigations apply mass spectrometric methodologies to the analysis of 3D cell cultures, many 3D cultures were homogenized for analysis in every study.

Colon cancer can be modeled with 3D cell cultures, referred to as spheroids. Spheroids can be generated from several different clonal cell lines. The immortalized colon carcinoma cell line HCT 116 grows readily to form reproducible spheroids when cultured on a bed of agarose. These spheroids grow to a diameter of about 1 millimeter after 14 days in culture and are very reproducible (Figure 1). In this manuscript, we demonstrate the first successful example of proteomic analysis of single 3D cell cultures by mass spectrometry.

Experimental

Chemicals and Reagents

Bovine pancreas TPCK-treated trypsin, agarose, formic acid, urea, ammonium bicarbonate (NH₄HCO₃), dithiothreitol (DTT), and iodoacetamide (IAA) were purchased from Sigma-

Aldrich (St. Louis, MO, USA); MS Grade Acetonitrile and MS Grade water were purchased from Honeywell – Burdick & Jackson (Muskegon, MI, USA). C18 SpinTips (product #89873) and 96-well plate were obtained from Thermo Scientific, and water was deionized by a Nano Pure system from Thermo Scientific (Marietta, OH, USA). McCoy's 5A media, glutamine, and FBS were purchased from Life Technologies – Gibco (Grand Island, NY, USA); HTC116 cells were purchased from the ATCC (Manassas, VA, USA). Mammalian Cell PE Lysis Buffer for spheroid lysis was obtained from G-Biosciences (St. Louis, MO, USA). Complete protease inhibitor mini tablets were purchased from Roche (Indianapolis, IN, USA).

Sample Preparation

The 3D HCT 116 multicellular culture growth was based on the methods reported by Li *et al*¹². Briefly, HCT 116 cells were cultured as a monolayer in a T25 flask at 37°C and 5% CO₂ in McCoy's 5A complete media with L-glutamine and 10% FBS. After adding trypsin to disrupt the monolayer, 6000 cells were seeded in the inner 60 wells of a 96-well plate, on top of a meniscus of agarose in each well. The 3D cultures were allowed to grow for 14 days with media changes every two days. Individual spheroids were visually verified via phase contrast microscopy to ensure that representative single spheroids were harvested. Micrographs of spheroids were obtained at harvesting time. Single 3D cultures were washed twice with 1X phospho-buffered saline to remove excess bovine serum and placed into separate 1.5 mL Eppendorf tubes. Cultures were frozen at –80° C until sample preparation.

Lysate preparation and sample digestion was referred to reference 13 with some modifications. 20 µL of commercial mammalian cell lysis buffer containing NP-40 supplemented with complete protease inhibitor was added to the cultures. The samples were sonicated at high frequency on ice with a Branson Sonifier for 15 minutes in 3 five-minute increments, until complete cell lysis was achieved. The lysates were incubated on ice for 30 minutes, to ensure protein solubilization. The lysates were centrifuged at 10,000 g for 10 minutes in an Eppendorf benchtop centrifuge at 4 °C to pellet cell debris. Separate samples were used for protein quantification and mass spectrometry analysis. Samples for protein quantification were diluted to 80 µL with nanopure water for the bicinchoninic acid (BCA) assay¹⁴. Protein quantification was performed according to manufacturer's instructions for microplates¹⁵. An 80 µL aliquot of cold acetone was added to the cell lysates for mass spectrometry analysis. Lysates were incubated at –20 °C overnight. The samples were centrifuged at 10,000 g for 10 minutes to pellet the samples. Acetone was removed via pipette, and the pellets were washed with 80 µL of cold acetone and rested at –20 °C for 30 minutes. Centrifugation was repeated. Then, the acetone was carefully removed with a pipette and the pellet was dried inside a chemical hood at room temperature for about 3 minutes, until all the acetone evaporated.

The protein pellet was dissolved in 10 µL 8M urea in 100 mM NH₄HCO₃ (pH 8.0) for one hour at 37° C. The proteins were reduced with 1 µL of 0.5 M DTT at 60° C for 30 minutes, then alkylated with 2.5 µL of 0.5 M IAA at room temperature for 30 minutes in the dark. Proteins were digested with TPCK-treated trypsin at 37° C overnight with a trypsin-to-protein ratio of 1:30. Samples were acidified to pH ~3 with 20% formic acid to quench

trypsin digestion, and each digest was desalted with C18 SpinTips. After drying, samples were resuspended in 15 μ L of 2% ACN/0.1% formic acid in water and analyzed via ultra-performance liquid chromatography/nano-electrospray ionization tandem mass spectrometry (UPLC/nESI-MS/MS).

UPLC/nESI-MS/MS analysis

A Waters nanoACQUITY Ultra-performance LC system (Milford, MA, USA) was used for separation of the protein digests. Buffer A (0.1% formic acid in water) and Buffer B (0.1% formic acid in acetonitrile) were used as the mobile phases for the separation. For each run, 1.5 μ L of spheroid peptides were loaded onto a C18 reverse-phase column (Waters, 100 μ m $\times$ 100 mm, 1.7 μ m particle size, BEH130C18) held at 40 $^{\circ}$ C, with 2% buffer B for 10 minutes at 1 μ L/min, followed by 90 minutes from 8% buffer B to 30% Buffer B at 0.6 μ L/min. The column was washed at 80% buffer B at 1 μ L/min, followed by equilibration for 12 minutes at 2% buffer B at 1 μ L/min. The total UPLC method time was 120 minutes for each run. The eluted peptides were pumped through a nano-ESI spray emitter and analyzed by the LTQ Orbitrap Velos instrument (Thermo Fisher Scientific, Waltham, MA, USA). The electrospray voltage was 1.8 kV. Each biological sample was run in technical duplicate.

The mass spectrometry method was referred to reference 16 with minor modifications. A top 20 data dependent acquisition (DDA) method was used. Full MS scans were acquired in the Orbitrap mass analyzer over m/z 350–1500 range with resolution 60,000 (m/z 400). The target value was 1.00E+06. Twenty most intense peaks with charge state 2 were selected for sequencing and fragmented in the ion trap with normalized collision energy of 35%, activation $q = 0.25$, activation time of 10 ms, isolation window of 2 m/z units and one microscan. The target value was 1.00E+04. The ion selection threshold was 500 counts, and the maximum allowed ion accumulation times were 500 ms for full scans and 100 ms for collisionally induced dissociation (CID). Dynamic exclusion was enabled, and peaks selected for fragmentation more than once within 20 seconds were excluded from selection for 60 seconds.

Data analysis

Proteome Discoverer software (Thermo Fisher Scientific, version 1.4) was used for data analysis. Mascot (version 2.2.4) and UniProt human database (number of sequences: 89,601) were used for database searching. The corresponding reversed database was also used for database search in order to evaluate the false discovery rates (FDRs) of identifications.

The original MS/MS spectra were first filtered with top 8 peaks per 100 Da mass windows, and then were subjected to Mascot search. Trypsin with full cleavage specificity and maximum missed cleavage sites as 2 were used for search. Precursor mass tolerance and fragment mass tolerance was 20 ppm and 1 Da, respectively. Carbamidomethylation (C) was set as static modification. Methionine oxidation, protein N-terminal and lysine acetylation and deamidation (NQ) were set as dynamic modifications. The database searching results were evaluated with Percolator (version 2.04) software. On peptide level, peptide confidence as high was used for filtering, and the corresponding peptide-level FDR was less than 1%. For peptide per protein, minimal number of peptide as 1, count only rank 1 peptides and

count peptide only in top scored proteins were applied. The protein grouping was enabled and strict maximum parsimony principle was applied. If multiple proteins were identified from the same peptides, they would be grouped into the same protein group. Each protein group has at least one unique peptide matching.

Analysis was also performed using MaxQuant¹⁷ (version 1.4.1.2), and Andromeda search engine was used for database searching against the UniProt human database containing forward and reversed sequences. The database also contains common contaminants. MaxQuant analysis included an initial search with a precursor mass tolerance of 20 ppm, main search precursor mass tolerance of 6 ppm and fragment mass tolerance of 0.5 Da, respectively. The search included enzyme as trypsin, variable modifications of methionine oxidation, protein N-terminal and lysine acetylation and deamidation (NQ), and fixed modification of carbamidomethyl cysteine. Minimal peptide length was set to seven amino acids and the maximum number of missed cleavages was set to two. The match-between-runs function was used. The label-free quantitation (LFQ) function was also enabled for the five biological replicate spheroids in order to quantitatively evaluate the protein expression abundance between single spheroids. The FDR was set to 0.01 for both peptide and protein identifications. The proteins identified by completely same sets of peptides were grouped, and reported as one protein group. The identification table was filtered to remove the identifications from the reverse database and common contaminants.

Results and Discussion

To our knowledge, this report represents the first published completion of proteomic analysis of single 3D cell cultures of any cell line. Previous reports have detailed mass spectrometric analysis of the proteomes of 3D cell culture spheroids^{18,19,20} but all of these included multiple spheroids, larger amounts of starting material, and/or larger on-column loading amounts. Many of these studies were proteomic comparisons of 2D and 3D cell culture.

A typical HCT116 spheroid contains ~ 40 µg of protein (Figure 2, Supplemental Table S-1). Several steps in the protocol were designed to minimize sample loss. Protein extraction using Nonidet P-40 (NP-40) lysis buffer requires an acetone precipitation to completely remove the detergents from the sample¹³. This procedure can cause sample loss, leaving even less protein for analysis. This loss can also selectively reduce the low-abundance proteins in the sample. Our procedure employed few pre-processing steps to minimize sample loss and improve identification of low-abundance species. As a single-tube protocol, the largest losses are expected to occur in the precipitation and desalting steps, with further possible losses upon drying and resuspension of the peptides, as well as some adsorption to the walls of the tube. However, the concentrations of the protein in solution was maintained at or above 1 µg/µL, in order to minimize possible sample losses²¹. Concentrations above 1 µg/µL are especially critical for the acetone precipitation step, where sample loss can be severe²². Higher initial sample concentration in an acetone precipitation prevents adsorption losses in both processing and desalting and allows for better efficiency of digestion.

In this study, two groups of replicate spheroids were used. One group of spheroids was used to assess total protein amount. A second group of replicate spheroids was used in the mass spectrometric analyses. The average value for total protein quantity for single spheroids was determined (Figure 2 and Supplemental Table S-1) with the BCA assay. An average value of $39 \pm 4 \mu\text{g}$ total protein was determined (Range: 37–47 μg) from 6 biological replicate spheroids. These spheroids had an average diameter of $940 \pm 30 \mu\text{m}$ (Range: 910–977 μm). The protein amount varies slightly among the cultures; images of the 3D cultures used to assess size and protein content are shown in Supplemental Figure S-1 (Panel A).

The average protein amount determined for single spheroids was used as an estimate for the starting value in the mass spectrometry protocol. For each spheroid, more than 1350 protein groups were reproducibly identified in duplicate runs (range 1356–1731 protein groups). The numbers of protein groups identified from each mass spectrometry experiment, as well as peptide and spectral data, are shown in Table 1. Micrographs of the spheroids used for mass spectrometry analysis are shown in Supplemental Figure S-1 (Panel B). A list of the protein groups identified from each spheroid is shown in Table S-2. Roughly equal numbers of tandem spectra were obtained from each experiment; the most variable data were the peptide spectral matches (PSMs), which affected the overall protein group identification.

Previous reports detail the proteomic profiles generated from mass-limited samples. For example, Wang et al¹³ published a study in which they analyzed small numbers of 2D cultured MCF-7 cancer cells, in pursuit of cell-typing the circulating tumor cells. Using a QTOF instrument, they identified an average of 619 ± 59 proteins from 5000 cells. Using LC-UV detection, they determined the mass of peptides in the sample to be $1.40 \pm 0.12 \mu\text{g}$. The authors state that 91% of the digested peptides were loaded onto the column, resulting in about 1 μg on-column digest. In the current study, we build on these results. The use of improved instrument and search algorithms generated nearly double the number of protein groups and peptide ID's from similar loading amounts of sample.

The mass spectrometry data obtained varies roughly 20% in terms of protein group identifications, despite size similarities. The MaxQuant label-free quantitation (MaxLFQ) method²³ was used to further investigate this variation. The LFQ intensities for each spheroid were filtered to remove contaminants and reversed-database identifications. The data was loaded into the Perseus analysis software (version 1.5.0.31)²⁴. The data was then sorted according to intensity, from largest to smallest. Any protein group not appearing in at least three of the spheroids was filtered out, leaving around 1400 protein groups. Pearson correlations between each sample were calculated and plotted (Supplemental Figure S-2). The Pearson correlation for each comparison was 0.962 or greater, showing a high degree of similarity in the identified protein groups between samples. Plots of the $\log_{10}$ combined LFQ intensity for all runs versus the $\log_2$ of the ratios of the LFQ intensities of each spheroid show centering around 0, similar to the method described by Cox, *et al.* (Supplemental Figure S-3, A–D). The ratios for the lower intensity groups appear more dissimilar compared to the highest intensities. The variation in identifications between runs is almost entirely due to stochastic variations in the fragmentation spectra; the samples themselves contribute very little to the variation observed.

Conclusions

This study provides a framework for single 3D cell culture proteomics. It is the first successful demonstration of mass spectrometric analysis of single 3D cell cultures. While 3D cell cultures are utilized in research due to their reproducibility and potential for unlimited sample expansion, individual cultures may not reflect the bulk average. Thus, while previous studies have interrogated homogenized groups of cultures, in this report, we investigated the proteomes of distinct cultures to determine if individual patterns are distinct from the bulk average. Individual spheroid analysis can be advantageous in the case of environmental and chemical insults, such as drug studies and nutrient variation in culture. As 3D cultures are excellent models of human organ and tumor growth, the ability to sample and interrogate these unique specimens has value.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Reproducibility of HCT 116 spheroid diameter. HCT 116 cells were seeded at three different cell densities and cultured for 21 days. Photographs were taken of each spheroid daily and measured for spheroid diameter. Each data point represents five measurements of six spheroids. The diameter of the spheroids varies less than 5% standard deviation for all three seeding densities.

Figure 2.

Spheroid protein quantification. Protein quantification was performed for each HCT 116 spheroid shown using the BCA method. The protein quantity is reproducible for spheroids of similar diameter. The protein quantity averaged $39 \pm 4 \mu\text{g}$ for a single spheroid. This estimate was applied to each spheroid for the bottom-up workflow and mass spectrometry analysis.

Table 1

Summary of results for mass spectrometry analysis. Data was analyzed using Proteome Discoverer 1.4 using a MASCOT search of the Uniprot Human database. Biological samples were run in duplicate.

Spheroid	MS/MS spectra	Peptide Spectral Matches	Peptides	Protein Groups
C3	24765	8914	6274	1536
	24553	8836	6277	1551
C11	24167	6981	5018	1361
	24446	7039	5037	1356
E10	25277	11088	7631	1732
	25404	11223	7785	1741
F5	24998	7384	5459	1449
	24320	7057	5262	1409
G9	25310	11620	8041	1751
	25145	11582	8021	1717