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Evaluation and Application of Dimethylated Amino Acids as Isobaric Tags for Quantitative Proteomics of the TGF- β /Smad3 Signaling Pathway

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Graphical abstract

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Supporting Information

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Scheme S1. Dimethylated amino acid reagent syntheses. Figure S1. Quantitative performance of DiLeu and DiAla with standard ratios. Figure S2. PSEA-Quant result comparison between two tags. Figure S3. DAVID analysis of protein candidates from DiAla (A) and DiLeu (B) comparing AdSmad3 to GFP. Table S1. Fractional purities of reporter ions of dimethylated amino acids. Table S2. Reporter ion m/z values (PDF)

Differentially expressed protein candidates identified with DiAla (XLSX)

Differentially expressed protein candidates identified with DiLeu (XLSX)

PSEA-Quant results with all quantifiable proteins from DiAla (XLSX)

PSEA-Quant results with all quantifiable proteins from DiLeu (XLSX)

DAVID analysis results with differentially regulated proteins from DiAla (XLSX)

DAVID analysis results with differentially regulated proteins from DiLeu (XLSX)

All protein identifications from MOVAS cells with DiAla (XLSX)

All protein identifications from MOVAS cells with DiLeu (XLSX)

Abstract

Isobaric labeling has become a widespread tool for quantitative proteomic studies. Here, we report the development and evaluation of several dimethylated amino acids as novel isobaric tags for quantitative proteomics. Four-plex dimethylated alanine (DiAla), valine (DiVal), and leucine (DiLeu) have been synthesized, sharing common features of peptide tagging and reporter ion production. DiAla and DiLeu are shown to achieve complete labeling. These two tags' impacts on peptide fragmentation and quantitation are further evaluated using HEK293 cell lysate. DiAla labeling generates more abundant backbone fragmentation whereas DiLeu labeling produces more intense reporter ions. Nonetheless, both tags enable accurate quantitative analysis of HEK293 cell proteomes. DiAla and DiLeu tags are then applied to study the TGF- β /Smad3 pathway with four differentially treated mouse vascular smooth muscle (MOVAS) cells. Our MS data reveal proteome-wide changes of AdSmad3 as compared to the GFP control, consistent with previous findings of causing smooth muscle cell (SMC) dedifferentiation.¹ Additionally, the other two novel mutations on the hub protein Smad3, Y226A, and D408H, show compromised TGF- β /Smad3-dependent gene transcription and reversed phenotypic switch. These results are further corroborated with Western blotting and demonstrate that the novel DiAla and DiLeu isobaric tagging reagents provide useful tools for multiplex quantitative proteomics.

Keywords

dimethylated amino acid; isobaric tag; smooth muscle cell; quantitative proteomics; phenotype

1. INTRODUCTION

Quantitative proteomics using mass spectrometry (MS) has gained its popularity in its application to the relative quantification of protein expression levels in different biological states. Quantitative MS approaches are either label-free^{2,3} or label-based.⁴⁻⁶ Labeling methods allow multiplexing of samples and enable comparative analysis in one LC-MS run. Thus, labeling can increase throughput, improve quantitative accuracy, and decrease run-to-run variability. Numerous MS-based chemical derivatization approaches have been reported and widely used for quantitative proteomics.⁶⁻⁸ Among them, isobaric labeling techniques have undergone rapid development in recent years due to their capability for increased multiplexing.⁶ In addition, the multiplexing capacity of these reagents allows for the

inclusion of more technical and biological replicates, providing greater statistical validation within any given experiment.⁹

Tandem mass tags (TMT)¹⁰ and isobaric tags for relative and absolute quantitation (iTRAQ)¹¹ are two major types of isobaric reagents that are commercially available. Our lab has developed a novel, cost-effective 4-plex set of dimethylated leucine (DiLeu) isobaric reagents and found its performance to be comparable to commercial reagents.^{12,13} Subsequent development provided an array of DiLeu-based reagents in an attempt to meet different research requirements for relative¹⁴ and absolute quantitation.¹⁵ In this work, we explore the development and application of other dimethylated amino acids for quantitative proteomics and compare their performance with the more established 4-plex DiLeu tags, in the context of restenosis.

Restenosis is a common adverse event of endovascular procedures that are used to treat the vascular damage from atherosclerosis. It is characterized by the recurrence of stenosis, a narrowing of a blood vessel, leading to restricted blood flow. It occurs in 30–50% of patients depending upon the artery treated, imposing a major limitation to the long-term success of surgical interventions.^{16,17}

Transforming growth factor beta (TGF- β) plays a prominent role in regulating a variety of cellular functions, including cell migration, cell proliferation, apoptosis, differentiation, immunosuppression, inflammation, tumor suppression, and angiogenesis.¹⁸ Abnormal TGF- β signaling also has been associated with several diseases,^{19,20} including restenosis.²¹ The prevailing view is that through its primary signal transduction mediator, Smad3, TGF- β stimulates vascular SMC intimal hyperplasia (IH), which is the main cause for restenosis. Activation of Smad3 by TGF- β stimulates IH and enhances adaptive remodeling. However, Smad3 serves as a hub protein for over 50 protein–protein interactions²² so that simply knocking out Smad3 is not a feasible treatment. Differentiating the TGF- β /Smad3 pathways that lead to adaptive remodeling versus IH will further clarify the underlying mechanisms behind restenosis as well as inform the design of inhibitors that have the ability to alter IH but not interfere with adaptive remodeling. Therefore, dissecting intertwined and probably overlapping binding sites on Smad3 is necessary to determine the specific pathways responsible for restenosis. Y226A and D408H are two mutations that can block or reverse Smad3 functions.^{23,24} These critical mutations provide ideal targets to achieve our desired goal.

In this work, we expand upon the original DiLeu isobaric tagging concept by synthesizing and testing two novel dimethylated amino acids (i.e., DiAla and DiVal) to provide more options in the amino-acid-based isobaric reagent toolbox. Based on our evaluation, DiAla and DiLeu tags offer complete labeling. We then evaluate their impacts on labeled peptide fragmentation and discover that DiAla tags produce more backbone fragments compared to DiLeu. However, they both offer comparable quantitative accuracy. Furthermore, the average cost of a set of 4-plex DiAla or DiLeu labels sufficient for a single experiment (100 μ g of protein digest per channel) is estimated to be about ten dollars, whereas the same amount of iTRAQ or TMT reagents would cost more than several hundred dollars, due to the complex, multistep syntheses required to produce these commercial reagents. The reduced cost for

reagent production with reliable quantitation accuracy makes amino-acid-based reagents an attractive alternative for quantitative proteomics. To demonstrate their utility in large-scale investigation of the systems biology problem, we employ these custom-synthesized tags in the proteome-wide analysis of changes caused by manipulation of Smad3 to help identify and establish the TGF- β /Smad3 induced signal transduction pathway and differentiate IH from remaining overlapping pathways.

2. EXPERIMENTAL PROCEDURES

2.1. Materials

Reagents for the synthesis of labels included the following (Scheme S1): all isotopic amino acids (L-leucine and L-leucine-1- ^{13}C , ^{15}N , L-alanine and L-alanine-1- ^{13}C , ^{15}N , L-valine and L-valine-1- ^{13}C , ^{15}N) and ^{18}O water 97% (H_2^{18}O) were purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA). Heavy formaldehyde (CD_2O), heavy water (D_2O), sodium cyanoborodeuteride (NaBD_3CN), sodium cyanoborohydride (NaBH_3CN), 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium tetrafluoroborate (DMTMM) and 1.0 M triethylammonium bicarbonate buffer (TEAB) were purchased from Sigma-Aldrich. *N*-Methylmorpholine (NMM) was purchased from TCI America (Tokyo, Japan). Protease (Cat. # 11836170001) and phosphatase inhibitor cocktail tablets (Cat. # 04906845001) were purchased from Roche. Tris base, urea, water, acetonitrile, and formic acid (FA) for UPLC were purchased from Fisher Scientific.

2.2. Synthesis of N,N-Dimethylated Amino Acids

Synthesis followed procedures published previously¹² with minor modifications (Scheme S1). Heavy isotopes, ^2H , ^{13}C , ^{15}N , ^{18}O , are strategically placed onto the structure to create three isobaric 4-plex reagent sets (Figure 1).

2.3. Cell Culture

HEK293 and MOVAS cells were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM media containing 10% FBS at 37 °C with 5% CO_2 as instructed. Biological triplicate MOVAS cells were then infected with retrovirus expressing wild-type or Smad3 point mutation (Y226A or D408H) and sorted by fluorescence activated cell sorting (FACS). The expression of wild-type or Smad3 mutants were confirmed by FACS and Western blotting. Biological triplicate MOVAS cells expressing GFP were used as control.

2.4. Enzymatic Protein Digestion

Proteins were dissolved in 8 M urea, reduced (5 mM DTT, 1 h at room temperature) and alkylated (15 mM IAA, 30 min at room temperature in the dark). Alkylation was capped by incubation in 5 mM DTT for 5 min at room temperature. Urea was adjusted to 0.9 M and trypsin was added in a 1:50 (w/w) ratio and incubated for 18 h at 37 °C. Each digest was quenched by the addition of TFA to a final concentration of 0.3%, desalted on a C_{18} SepPak cartridge (Waters, Milford, MA), and dried under vacuum. Protein digests were then redissolved in 0.5 M TEAB before labeling.

2.5. Activation of DiAla, DiVal, and DiLeu, and Protein Digest Labeling

DiLeu tags were suspended in anhydrous DMF and combined with DMTMM and NMM at 0.6× molar ratios to DiLeu. The mixture was then vortexed at room temperature for 60 min. Following centrifugation, the supernatant was immediately mixed with protein digest. Peptides were labeled by adding the activated label solution at a 10:1 label to peptide digest ratio by weight and vortexing at room temperature for 2 h. The reaction was quenched by adding 5% NH_2OH to the final concentration of 0.25% and dried under SpeedVac. Peptides labeled with different channels were then mixed with equal amounts or experiment-specific ratios (i.e., 8:4:2:1 and 1:2:4:8).

2.6. Strong Cation Exchange (SCX) Fractionation

Peptides labeled with different isotopic variants of isobaric tags were mixed together and resuspended in 100 μL of 75% 10 mM ammonium formate, 25% acetonitrile, pH 3.0 with formic acid and fractionated by strong cation exchange (SCX) liquid chromatography using a PolySULFOETHYL A column (200 mm $\times$ 2.1 mm, 5 μm , 300 Å, PolyLC, Columbia, MD). Mobile phase A consisted of 75% 10 mM ammonium formate, 25% acetonitrile, pH 3.0 with formic acid, and mobile phase B was 75% 500 mM ammonium formate, 25% acetonitrile, pH 6.8. The column was initially loaded and washed for 20 min with 0% B. Peptides were eluted using a linear gradient of 0–50% B over 50 min and then increased to 100% over 10 min. The column was subsequently washed at 100% B for an additional 10 min all at a flow rate of 0.2 mL/min. The column effluent was monitored at 280 nm with a Waters 2489 UV/Visible detector, and fractions were collected with a FC-4 fraction collector (Rainin Dynamax) followed by drying under SpeedVac.

2.7. Reversed-Phase Separation and Mass Spectrometry

2.7.1. Normalized Collision Energy (NCE) Optimization—NCE ramp tests were conducted by operating the Orbitrap Elite at a MS resolution of 30K, MS² resolution of 15K, and an isolation width of 2 Th. The most abundant ion from m/z 350–1500 in each cycle was isolated 10 times and fragmented with HCD normalized collision energies (NCEs) of 10, 15, 20, 25, 27, 30, 32, 35, 40, and 45. The Coon OMSSA (Open Mass Spectrometry Search Algorithm) Proteomic Analysis Software Suite (COMPASS)²⁵ was used for data analysis by HCD energy comparisons.

2.7.2. Fragmentation Comparison between DiAla and DiLeu—Four $\times 50 \mu\text{g}$ of peptides labeled with 4-plex DiAla and 4-plex DiLeu were mixed together and fractionated. Peptide identification by liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis was performed using a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) with a NanoAquity UPLC system (Waters). Peptides were dissolved in 40 μL of 0.1% FA, out of which 3 μL was loaded and separated on a 75 $\mu\text{m} \times 15$ cm homemade column packed with 1.7 μm , 150 Å, BEH C18 material obtained from a Waters UPLC column (part no. 186004661).

2.7.3. MOVAS Protein Identification and Quantification—For each biological replicate, 4 $\times 50 \mu\text{g}$ of peptide digest from 4 samples were labeled with 4-plex DiAla or 4-plex DiLeu and fractionated. Peptide identification by LC/MS/MS analysis was performed

using an Orbitrap Elite Ion Trap-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) interfaced with a NanoAquity UPLC system (Waters). Peptides from each fraction were dissolved in 40 μL of 0.1% FA, out of which 3 μL was loaded and separated on a 75 $\mu\text{m} \times 15$ cm self-fabricated column packed with 1.7 μm , 150 Å, BEH C18 material obtained from a Waters UPLC column (part no. 186004661).

2.8. Western Blot

Cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP40, 0.1% sodium dodecyl sulfate) with protease inhibitor cocktail (Roche). Forty micrograms of protein from each sample was separated on SDS-PAGE, and then transferred to PVDF membranes. Proteins on transferred membrane were assessed by antibodies to smooth muscle alpha actin (SMA), calponin and myosin heavy chain. After washing with TTBS and incubated with horseradish peroxidase-conjugated secondary antibodies, the PVDF membrane was developed with chemiluminescence substrate (Fisher Scientific, Davenport IL) and the images were acquired with Fujifilm Imager 4000mini.

2.9. Data Analysis

Mass spectra were processed using Proteome Discoverer (version 1.4.0.288, Thermo Scientific). Raw files were searched in Proteome Discover against the UniProt *H. sapiens* or *M. musculus* complete database using the Sequest HT algorithm. Trypsin was selected as the enzyme with two missed cleavages allowed. Sequest HT was searched with a parent ion tolerance of 50 ppm and a fragment ion mass tolerance of 0.02 Da. Peptide spectral matches (PSMs) were validated based on q-values to 1% FDR (false discovery rate) using percolator. Quantitation was performed in Proteome Discoverer with a reporter ion integration tolerance of 20 ppm for the most confident centroid. Only the PSMs that contained all reporter ion channels were considered, and protein quantitative ratios were determined using a minimum of one unique quantified peptide. Reporter ion ratio values for protein groups were exported to Excel workbook and corrections were performed followed by the Student *t* test, which was performed with biological triplicates. The grand average hydrophobicity (GRAVY) values were calculated by the GRAVY calculator (<http://www.gravy-calculator.de/>).

2.9.1. Labeling Efficiency—Static modifications consisted of carbamidomethylation of cysteine residues (+57.0215 Da). Dynamic modifications consisted of isobaric labels on peptide N-termini, lysine residues (103.0833 Da for DiAla, 131.1146 Da for DiVal and 145.1303 for DiLeu) and oxidation of methionine residues (+15.9949 Da).

2.9.2. HEK293 and MOVAS Protein Identification and Quantitation—Static modifications consisted of carbamidomethylation of cysteine residues (+57.0215 Da), isobaric labels on peptide N-termini and lysine residues. Dynamic modifications was set to be oxidation of methionine residues (+15.9949 Da).

2.9.3. GO-Term Enrichment Analysis—Gene ontology (GO) enrichment analysis of the differentially expressed proteins by both tags was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.7.²⁶ Gene groups with enrichment scores ≥ 1.3 , which is similar to $P < 0.05$, were explored. Protein set enrichment

analysis (PSEA-Quant) was further used to scrutinize the entire protein quantification data set.²⁷ Abundance ratios were input into the online PSEA-Quant interface. The Gene Ontology annotation database was selected, protein abundance dependence was assumed, a coefficient of variation tolerance factor of 0.5 was input, and protein annotation bias was also assumed.

3. RESULTS

Our goal in this study is to examine newly designed and synthesized dimethylated amino acids as isobaric labeling reagents and evaluate their performance in large-scale analyses of complex biological samples. To this end, we synthesized three sets of novel 4-plex dimethylated amino acid isobaric tags and compared their labeling efficiencies. Two out of three (DiAla and DiLeu) achieved complete labeling and were selected for further characterization of their impacts on peptide fragmentation behavior and protein identification and quantitation. Tryptic HEK293 cell peptides were labeled with DiAla and DiLeu and mixed together for MS to eliminate systematic or run-to-run variations. After data-dependent acquisition (DDA), we only selected subset of peptides identified with both DiAla and DiLeu for further analysis. Subsequently, we employed these two tags to study TGF- β /Smad3 related mechanisms underlying intimal hyperplasia.

3.1. Characteristics of 4-Plex Isobaric Labeling Reagents

Covalent bonding of these three isobaric labeling reagents to peptide N-termini and lysine residues adds relatively modest masses to each site labeled. Attached tags fragment into dimethyl immonium reporter ions with distinct m/z values with one Dalton intervals upon HCD or collisional induced dissociation (CID) (Table S2).

3.2. Comparison of Isobaric Labeling Efficiency and Collision Energy Optimization

The labeling efficiency of three isobaric reagents were assessed by setting tags as dynamic modifications and calculating the percentage of labeled N-terminus and lysine residues (Figure 2). All three labeling reagents have the same reactive group and are similar in sizes, however, their labeling efficiencies to amine group vary from each other. DiAla and DiLeu rendered ~100% labeling completeness whereas DiVal only labeled ~87% available sites. This relatively low labeling efficiency by DiVal is likely attributed to the steric hindrance, imposed by isopropyl group at its β -carbon atom, against nucleophilic attack from primary amines to accomplish the substitution.^{28–30} This phenomenon has also been observed in a variety of substitution reactions involving valine,^{29,31–33} one of the sterically hindered amino acids.

We compared the numbers of PSMs generated with various HCD NCEs fragmenting HEK293 tryptic peptides labeled with DiAla and DiVal by using an Orbitrap Elite mass spectrometer. The most abundant precursor ion from each MS spectrum was isolated and its MS² spectra acquired 10 times at HCD NCEs of 10, 15, 20, 25, 27, 30, 32, 35, 40, and 45. The maximum number of target PSMs was achieved at NCE 27 for DiAla and NCE 30 for DiVal, as displayed in Figure 2B. It was reported previously that NCE 27 was also the optimal NCE for DiLeu.^{14,15}

3.3. Isotopic Impurity Determination

As is common with all stable isotope labeling reagents, the purities of the reporter groups are less than 100% because isotopic starting materials used in the reagent syntheses contain less than 100% stable isotopes.³⁴ As a result, each primary reporter ion peak is accompanied by low-intensity isotopic impurity peaks that are greater or lesser in mass by one neutron. To generate an accurate quantitative result, the fractional contribution of each channel's primary reporter ion peak and those of isotopic peaks were determined with a 20 ppm mass tolerance (Table S1). Corrections were made according to the fractional purities in the subsequent quantitative studies.

3.4. Comparison of Isobaric Labeling Reagents' Impact on Peptide Identification

Derivatization of a peptide influences its fragmentation pattern. The tag increases the mass distribution of the sample and changes the charge distribution. Sulfonic acid derivatives were shown to fragment more efficiently in MS² than the native peptides.³⁵ Hsu et al. reported a dimethyl labeling approach for improved identification of tryptic peptides, including the production of an enhanced a1 ion for *de novo* sequencing.^{8,36} iTRAQ and TMT were also reported to alter peptide charge states and identification performance.^{37,38}

DiAla and DiLeu, the two reagents that can deliver ~100% labeling efficiency, were selected to compare how the label affected peptide fragmentation behaviors and subsequent identifications. In a duplicate experiment for an unfractionated proteome with a Top15 HCD method, DiAla-labeled samples resulted in 60450 averaged total MS² spectra, whereas DiLeu-labeled samples yielded 57123 tandem MS spectra. By searching the data, we found that DiAla-labeled samples always generated more protein identifications, peptide identifications, and especially PSMs (Figure 3A). This observation suggested that the two tags can alter peptide fragmentation to different degrees despite the similar small size of these two tags. To investigate this situation further, equal amounts of labeled HEK293 peptides from both tags were mixed together and analyzed with various numbers of SCX fractions. DiAla tagging consistently generated more identifications (Figure 3B). Peptides were included in subsequent comparisons only if they were identified with both tags, called shared peptides. Several key characteristics were evaluated with a custom C# script. The first thing we examined was precursor ion intensity. Our results suggested that DiLeu derivatives ionized better compared to their DiAla counterparts, by producing higher precursor intensities (Figure 4A). It was previously reported by Kulevich et al. that increasing hydrophobicity through alkylation improves ionization and, within a proper range, higher hydrophobicity usually results in better ionization.³⁹ DiLeu has larger side chains and therefore is supposed to be more hydrophobic. Interestingly, if the hydrophobicity is the only difference between the two tags, DiLeu derivatized peptides are expected to result in more identifications due to the stochastic nature of a regular DDA method. However, this was not what we observed. DiAla-tagged peptides tended to produce more b-type and y-type fragment ions (Figure 4B and 4C). DiAla peptides produced 14.3 fragment ions on average, whereas that number for DiLeu peptides was 13.7, with a student *t* test *p*-value <0.05. Correspondingly, it was as expected that DiAla peptides had a higher percentage of theoretical fragments recorded by MS² scans (48.2% versus 46.2%). Since there is a balance between the production of reporter ions and backbone cleavage fragment ions, we evaluated

how the balances shift and vary between these two tags. Reporter ion intensities were averaged, and not surprisingly, DiLeu manifested higher values compared to DiAla peptides (Figure 4D). In another word, DiAla-labeled peptides give rise to more preferential cleavages along the peptide bonds, as revealed by the higher relative average backbone fragment intensity for shared peptides, which was calculated as the following expression:

$$\frac{\text{Averaged backbone fragment intensity}}{\text{Base peak intensity (BPI) in MS}^2} \times 100\%$$

The base peak in a MS² spectrum is always the reporter ion peak. DiAla had backbone fragments, on average, reaching 7.76% of its reporter intensity, whereas DiLeu had 5.56%. Another direct proof of its better backbone fragmentation facilitating database search is that DiAla-labeled PSMs had an averaged Sequest XCorr value at 3.2 whereas DiLeu had such value at 2.8. However, increased backbone fragment intensity did not lead to reduced DiAla reporter ion intensity and negatively impact the quantitation accuracy. Although the reporter signal is on average 2-fold less than that of DiLeu, the approximate ion intensity at about 1×10^6 still provided sufficient quantitative information. GRAVY values of unique identifications from each tag were further analyzed in their unmodified form (Figure 4F). As presented by the histogram, unique peptides from DiAla favors a bit more hydrophilicity, providing complementary coverage as compared to DiLeu. To summarize, DiAla and DiLeu, two small amino-acid-based labeling reagents, are capable of changing peptide hydrophobicity and fragmentation behaviors. Even though DiLeu, being more hydrophobic, increases ionization to a greater extent than DiAla, the latter tends to produce more intense backbone cleavage fragment ions that are important for peptide identification via database searching, as represented by a tryptic peptide TAFQEALDAAGDK (Figure 5).

3.5. Performance of 4-Plex DiAla and DiLeu in a Study of TGF- β /Smad3-Dependent Pathways

Three biological replicates, each of which contained peptides from four different MOVAS cell lines, were labeled with three sets of 4-plex labeling reagents. After SCX fractionation, peptides were combined into 5 vials, and aliquots from each of these vials were injected twice for LC-MS² analysis. Proteome Discoverer was used to combine data from all the LC-MS² runs, resulting in the identification of 3576 proteins from DiAla-labeled samples and 3886 from DiLeu-labeled samples at a peptide FDR = 1% (Figure 6), and the majority (~80%) of these proteins were identified using both tags. Proteins matched by at least one unique peptide had to be detected in all quantitation channels and replicates for quantification, which provided confident quantitative information on 2194 proteins from 4-plex DiAla and 2234 proteins from 4-plex DiLeu. Student *t* tests were performed between each pair of four gene types, resulting in six pairs of quantitative comparisons. The $-\log_{10}$ p-value of each protein was plotted against $\log_2$ protein ratio to construct a volcano plot measuring the statistical significance and magnitude of fold change for each protein (Figure 7). Four out of six had similar statistical distributions (Figure 7A–D); therefore, the same p-value ($p < 0.01$) and fold change (greater than 2) were set to be the statistical cutoff value. The other two featured much tighter distributions and were more similar in terms of the overall proteome landscape compared to the previous four pairs. A more relaxed criterion

was thereby used for these two pairs ($p < 0.05$ and fold change $> 20\%$) in order to pull out a proper number of representative protein candidates. Protein candidate numbers from each pair of comparisons are listed in Table 1. About half of these proteins exhibited overlaps between two tags, while the rest were unique to either DiAla or DiLeu based on statistically significant differences. These numbers were further evaluated. For example, between DiAla and DiLeu data sets for AdSmad3 and Y226A mutants, there were 75 protein candidates that passed both statistical filters, while 117 were unique to DiAla and 85 to DiLeu only (Figure 8A). We sorted this 117 into two subcategories, namely unique DiAla identifications, including 72 proteins that were not identified in the DiLeu data set, and significant proteins only from DiAla, including the remaining 45 proteins that were in the DiLeu data set but simply did not fulfill the statistical requirements. Similarly, 85 proteins that were identified by DiLeu tags consisted of 14 unique identifications with the remaining 71 statistically significant only using DiLeu labels (Figure 8A). Unique identifications to specific tags were complementary to each other and improved overall proteomic coverage. Except for the unique identifications, we also investigated if all other protein candidates identified from both data sets had similar fold changes reflected by their reporter ion ratios. To examine this question, DiAla fold changes were plotted against the DiLeu fold changes of the shared 75 candidates (Figure 8B). Each dot represents one protein, and they all fall along the $y = x$ line, suggesting that the two tags offered consistency in quantitation with excellent correlation. The fold changes observed for protein candidates with only one tag showing significant changes were plotted with DiAla tags on the y -axis against those of DiLeu tags on the x -axis. They exhibited the same general trend (Figure 8C). 40% of these candidates that stood out in one data set but not the other were within 10% range of the 2-fold change threshold (0.4–0.5 or 2–2.2), and most of their counterparts were very close to the threshold. Proteins with the same trend but showing different fold changes are likely due to coisolation of interfering peptide precursors.^{6,40–43} Such interference can be further minimized with various approaches upon availability of certain instruments in the future.^{40–42} In summary, DiAla and DiLeu tags were able to generate consistent quantitation data, and to some extent, they complemented each other by providing expanded proteome coverage.

To analyze our data set further, we performed a GO term analysis. While traditional DAVID analysis focuses on proteins that pass an arbitrary t test threshold,²⁶ PSEA-Quant considers the entire data set and complements DAVID analysis by providing a more global view. When we applied both types of analysis to our data set, several GO-terms, many potentially related to restenosis, were enriched. The side-by-side comparison between AdSmad3 and GFP control using PSEA-Quant revealed 76 up-regulated terms, of which 21 were identified to be enriched using both tags, and 111 down-regulated ones, including 31 shared terms using both tags (FDR < 0.1) (Figure S2).

The vascular SMC in adult animals is a type of highly specialized cell whose principal function is contraction and regulation of blood vessel tone-diameter, blood pressure, and blood flow distribution.⁴⁴ SMCs within adult blood vessels undergo minimal proliferations and exhibit very low synthetic activity. However, in response to vascular injury or during the process of restenosis, the SMC dramatically increases its rate of cell proliferation and thereby dedifferentiates from a contractile to synthetic phenotype. TGF- β is one of the factors responsible for this phenotypic modulation and subsequent neointimal formation via

Smad3. It enhances intimal hyperplasia by stimulating extracellular matrix (ECM) synthesis.⁴⁵ Several major constituents of the ECM, for example elastin (ELN) and type I and type III collagens (COL3A1, COL1A2), of which TGF- β is a potent stimulator, modulate cell shape and proliferative responses and, thereby, contribute to the pathogenesis of intimal hyperplasia.^{46,47} They were all found to have enhanced expression in our data sets when comparing AdSmad3 and GFP control. Meanwhile, synthesis increase of another important multifunctional ECM glycoprotein, fibronectin (FN1), has been associated with synthetic SMCs during atherosclerosis and restenosis through a Smad3 signaling pathway activated by TGF- β .^{27,48}

In both of our data sets, we observed consistent changes of all these established molecules and the data agree well with each other (Tables S3 and S4). For example, COL3A1 exhibited 1.8-fold and 2.0-fold up-regulation compared to control using DiAla and DiLeu isobaric tags, respectively. FN1 exhibited even better consistency with both tags, suggesting 1.5-fold up-regulation. DiAla tagging showed complementary results to those obtained using DiLeu tags, revealing elevated expression of COL1A2 and ELN, as expected from previous studies. At the same time, DAVID and PSEA-Quant analysis revealed corresponding enriched GO terms (e.g., extracellular matrix structural constituent, fibrillar collagen) that clustered all relevant proteins together (Figure S3, Tables S5–S8). Other GO terms including endoplasmic reticulum, translation, and translation factor activity all indicated a much more synthetic phenotype compared to the GFP control sample.

In contrast, with the contractile phenotype disappearing, some signature proteins involved in the regulation of contraction of the SMC decreased, including multiple tropomyosins (TPM1, TPM2, TPM3)⁴⁹ and calponin (CNN1).^{1,50} We observed that several isoforms of these proteins had a consistent decreasing trend (Figure 9 and Tables S3 and S4). Myosin regulatory subunit (MYL9), a crucial element of the contractile apparatus,⁵¹ followed the same pattern, being down-regulated. Laminin (LAMC1), another adhesive glycoprotein and a component of the basement membrane, has been found to maintain the cells in a contractile phenotype⁵² and was shown to decrease in our MS-based proteomic analysis. In the normal state, unstimulated SMCs are nonmigratory because they are not proliferating and because the matrix is highly adhesive. However, during restenosis development, SMC proliferates and migrates into the intima, which calls for remodeling of the cytoskeleton as well as change of the adhesiveness of the cell to the matrix,^{53,54} as captured by our gene ontology analysis. Both DAVID and PSEA-Quant outputs revealed corresponding changes from a gene ontology point of view (Figure S3 and Tables S5–S8) by capturing GO terms such as actin cytoskeleton, contractile fiber, and regulation of cell motion. These factors would eventually render the SMCs unable to exert contractile responses and limit their regular function in maintaining blood pressure.

3.6. Y226A and D408H Have Different Influences on TGF- β Smad3 Induced Responses

Upon validating our tags with AdSmad3 and GFP, we explored the utility of these tags for quantifying proteomic changes of SMCs in two newly reported mutations, Y226A and D408H. Y226A mutation reduces binding to SARA and Ski, two major Smad-interacting proteins, and has severe reduction of the TGF- β -inducibility of endogenous genes.²⁴ D408H

is defective in binding to Smad4, the major binding partner of Smad3 and with whom Smad3 cotranslocates into the nucleus.²⁴ Compared to the GFP control and the AdSmad3, data from DiAla and DiLeu labeling suggested that Y226A and D408H were effective inhibitors of SMC migration and ECM constituent synthesis, and they were also able to tune down contractile apparatus expression, thereby attenuating SMC differentiation (Figure 9 and Tables S3 and S4). However, Y226A and D408H boosted the generation of precursor metabolite and energy, DNA replication, and cell division.

The comparison of Y226A against D408H indicated that D408H mutant was more thoroughly blocking the ECM constituent synthesis and shifted more resources on DNA replication, whereas Y226A still retained partial responses to TGF- β stimulus. Y226A revealed higher collagen, fibronectin, and laminin levels. On the other hand, DNA replication licensing factors MCM2, 4, 6, and 7 were found up-regulated in D408H (Tables S3 and S4). They act as components of the MCM2–7 complex. In eukaryotes, the MCM2–7 complex forms the core of the replicative helicase—the molecular motor that initiates replication and uses ATP binding and hydrolysis to fuel the unwinding of double-stranded DNA at the replication fork.^{55,56} Proliferating cell nuclear antigen (PCNA) was another overexpressed protein. It acts as a homotrimer and helps increase the processivity of leading strand synthesis during DNA replication by recruiting crucial players to the replication fork.⁵⁷ Meanwhile, cell respiration was also enhanced, as implicated by the increase in proteins including HK2, PGK1, GAPDH, ENO1, and TPI1,⁵⁸ all of which are involved in glycolysis.

4. DISCUSSION

In this study, we tested two newly designed and synthesized dimethylated amino acids as isobaric labeling reagents and systemically compared DiAla with our previously developed DiLeu tags. We labeled HEK293 cell lysates with DiAla, DiVal, and DiLeu, respectively, and compared the ability of each of these three tags to achieve labeling completeness, which is an absolute requirement for quantitative proteomics. We eliminated DiVal for further evaluation due to its inability to render complete labeling. We found that DiAla generated more peptide identifications compared to DiLeu. To fully characterize DiAla and DiLeu and to make a fair comparison, we pooled the same amount of DiAla and DiLeu labeled peptides together and only included peptides that were identified with both tags. We analyzed their ionization and fragmentation behavior.

As demonstrated, DiAla peptides, being generally less hydrophobic, do not ionize as well as DiLeu peptides during electrospray ionization. This is in good agreement with numerous previous studies on how peptide alkylation could affect ionization. It has been shown that, in general, more hydrophobic analytes produce larger ESI responses, which is attributable to greater surface affinity. Hydrophobic molecules are driven to the surface in an attempt to minimize interactions between the hydrophobic molecules and water and increase water–water interactions. Meanwhile, charges produced by ESI tend to distribute on the droplet surface, making it easier for hydrophobic molecules to obtain in comparison to hydrophilic analytes that reside deep inside the droplet.^{39,59} Also, leucine possesses higher proton affinity and gas-phase basicity, which also contributes to its better ionization efficiency.^{60–62}

However, this advantage does not make DiLeu superior to DiAla in terms of increasing proteome coverage. In general, we found that, for the same peptide, DiAla provided more backbone cleavage fragment ion peaks in higher abundance and lower reporter ion intensities. Due to the nature and scoring algorithm of most of the database search engines, it is more likely for DiAla-labeled peptides to acquire higher scores and therefore pass the FDR filter with ease. DiLeu peptides' production of more intense immonium a1 reporter ions when undergoing MS² dissociation can be partially explained by the two additional methyl groups on the leucine side chain, which are electron donating groups that can stabilize the positive charge on the immonium ion.^{12,63} Therefore, we conclude that DiAla is relatively ideal for peptide identification compared to DiLeu due to its ability to generate more backbone fragments, whereas DiLeu could provide more reliable quantitative results even for lower abundance proteins due to its inherent production of more intense reporter ions than other isobaric tags.

To emulate a representative biological experiment, we analyzed changes in the proteome from different MOVAS cell lines, attempting to evaluate and validate these tags' performance and applicability while elucidating the molecular underpinnings of the TGF- β /Smad-3 induced signaling pathways that enabled differentiation of intimal hyperplasia and adaptive remodeling. We first compared AdSmad3 to GFP control, which has been investigated previously. Consistent with previous findings,^{1,44,54,64} we observed enhancement of intimal hyperplasia and increased SMC proliferation by examining differentially regulated protein candidates as well as performing gene ontology analysis. Cells underwent dedifferentiation upon infection with AdSmad3 and TGF- β treatment, which was evidenced by a phenotype switch toward a synthetic phenotype rather than maintaining its contractile capability. These experiments confirmed that TGF- β /Smad3 stimulated rather than inhibited SMC proliferation.⁶⁵ In addition to validating the quantitative performance of the DiAla and DiLeu tags with GFP control and AdSmad3 cell lines, two novel mutants were included to investigate their impacts on TGF- β /Smad3 pathways. Y226A and D408H are mutations in different protein-binding hot-spots that have the potential to only perturb a subset of the total binding interactions, providing an opportunity to evaluate the biological significance of those interactions. Our results suggested that Y226A and D408H reduced SMC migration, ECM accumulation, and contractile capability to various extents. D408H had more thorough impacts, blocking these activities while maintaining a high rate of DNA replication, whereas Y226A retained moderately more ECM synthesis activities with lower DNA replication.

In summary, we show that DiAla and DiLeu are viable isobaric tags that enable simultaneous identification and quantification of a large number of proteins in complex biological systems. DiAla exhibits more efficient backbone fragmentation and enhanced sensitivity, rendering more identifications. DiAla and DiLeu are able to generate consistent quantification results and complement each other by increasing overall proteome coverage. Their utilities are validated by quantitatively comparing GFP and AdSmad3, which mimic the healthy and restenotic processes. The phenotypic switch is demonstrated by corresponding proteomic changes, and a few proteins that play vital roles in such a process are confirmed. In addition, Y226A and D408H, two novel mutants that are able to reverse

restenotic processes, reveal different capabilities in modulating downstream responses and therefore provide tools to dissect TGF- β /Smad3 signaling pathways for future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Dimethylated amino acids. Stable isotopes (^{13}C , ^2H , and ^{15}N) incorporated into the reporter group are mass-balanced by stable isotopes (^{13}C , ^{18}O) in the carbonyl balance group. Unique combinations of isotopes incorporated into the reporter group yield 4-plex DiAla, DiVal, and DiLeu.

Figure 2. Labeling efficiency (A) and collision energy optimization (B). (A) Labeling efficiency of DiAla, DiVal, and DiLeu. Bar plots represent the mean of three replicates $\pm$ SD. (B) Number of PSMs for the identified DiAla- and DiVal-labeled HEK293 tryptic peptides according to NCEs. Bar plots represent the mean of duplicates $\pm$ SD.

Figure 3. Identification comparison between DiAla and DiLeu from separate LC-MS experiments. (A) Identification comparison between DiAla and DiLeu from two separate single-shot experiments. The vertical axis on the left is used for protein groups whereas the secondary vertical axis on the right side is for peptide IDs and peptide PSMs. All values represent the mean of duplicates $\pm$ SD. (B) PSM comparisons with different numbers of SCX fractions of equal amounts of DiAla and DiLeu HEK293 tryptic peptide mixtures. PSMs were generated in three separate experiments with ascending numbers of SCX fractions collected, intended for better separation.

Figure 4.

Fragmentation behavior comparison between peptides labeled with DiAla and DiLeu. (A) Average precursor intensity at the time point where MS² was collected. (B) Average number of fragments recorded for shared peptides. (C) Average percentage of fragments recorded for shared peptides. (D) Average reporter intensity for shared peptides. (E) Average relative backbone fragment intensity for shared peptides. (F) Gravy value distribution of unique DiAla or DiLeu peptides. Values represent the mean $\pm$ SD. Results were statistically tested with a *t* test *p*-value of 0.05.

Figure 5. Spectra of DiLeu and DiAla labeled TAFQEALDAAGDK. The starred peak in each spectrum is the reporter ion. Area occupied by product ions is shaded in blue (left, DiLeu) or pink (right, DiAla).

Figure 6. Protein identification from MOVAS cells with DiAla and DiLeu. (A) Venn diagram representing the comparison of the proteins detected with DiAla and DiLeu. (B) PSM identified with two tags. Values represent the mean of three biological replicates $\pm$ SD.

Figure 7.

Overlaid volcano plots of quantified MOVAS proteins with DiAla (blue dots) and DiLeu (red dots). The $-\log_{10}$ p-value of each protein is plotted against $\log_2$ protein fold change. Proteins falling into the orange area are statistically down-regulated whereas those ones in the green shaded area are up-regulated. Proteins with fold-changes ≥ 2 and p-values < 0.01 are presumably differentially regulated in comparing AdSmad3 with Y226A mutant (A), AdSmad3 with D408H mutant (B), GFP control with Y226A mutant (C), and GFP control with D408H mutant (D). Proteins with fold changes of 20% and p-values < 0.05 are presumed to be differentially regulated proteins in comparing Y226A with D408H (E) and GFP control with AdSmad3 (F). Each data set is further characterized through PSEA-Quant.

Figure 8.

Representative quantitation result comparison between two tags using the data sets comparing AdSmad3 to Y226A mutant. Protein candidates are classified into several categories. (A) Two tags generate 75 shared protein candidates, with another 117 candidates from DiAla, consisting of 72 unique DiAla IDs plus 45 proteins that are statistically significant only from DiAla tagging. DiLeu labeling produces 85 unique candidates, and they consist of 14 unique IDs plus 71 proteins that are statistically significant only from DiLeu. (B) DiAla fold changes against DiLeu fold changes of the shared protein candidates. The solid line is $y = x$, and dots in the green area are up-regulated while those in orange are down-regulated. (C) DiAla fold changes against DiLeu fold changes of the protein candidates that are only significant with one tag. The solid line is $y = x$.

Figure 9.

Western blotting confirmed several proteins affected by Smad3 mutants in MOVAS cells. Proteins were extracted from cultured MOVAS cells, and Western blotting was performed using antibodies as indicated. The results showed that Smad3 mutants affect several smooth muscle cell protein expression levels in a similar trend as revealed by multiplexed quantification using DiAla and DiLeu tags.

Table 1

Protein Numbers Detected To Be Differentially Regulated from DiAla (blue), DiLeu (pink), and Their Overlaps (light yellow)^a

		DiAla			DiLeu			Shared		
			↑	↓		↑	↓		↑	↓
Differentially Expressed Protein Candidates p<0.01, fold change ≥ 2	Smad3	DiAla			DiLeu			Shared		
	Smad3 Y226A		148	44		132	28		71	4
	Smad3									
	Smad3 D408H		116	36		113	19		67	4
	GFP Control									
p<0.05, fold change ≥ 20%	Smad3 Y226A	DiAla	147	50	DiLeu	158	33	Shared	90	6
	GFP Control									
	Smad3 D408H		127	41		153	39		80	5
	Smad3									
	Smad3 Y226A	DiAla	67	45	DiLeu	88	43	Shared	35	16
	Smad3 D408H									
	GFP Control									
	Smad3		43	46		53	59		10	13

^a“↑” designates up-regulated protein candidates while “↓” designates down-regulated protein candidates.