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Editorial

The Human Proteome: 90% in the Light, 10% on the Dark Side

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DARK TIMES

2020 has been quite the year—dark to the point of a little less than awful. The first global pandemic in a century, a global recession, worsening global warming, and global audience angst before and during the American election—all deeply concerning. Yet there has been some light. 2020 is the tenth anniversary of the Human Proteome Project (HPP)[1] of the Human Proteome Organization (HUPO). Moreover, on October 19, 2020, the completion of the high stringency draft of the human proteome was announced in the opening session of the 19th HUPO virtual World Congress by the first Chair of the HPP, Dr. Gil Omenn. The formal reporting of this HPP success occurred concurrently in the HPP Consortium's *Nature Communications* paper by Adhikari et al.,[2] "A High-Stringency Blueprint of the Human Proteome". The depth of this accomplishment was reflected by the significant contributions of ∼500 contributors acknowledged in the paper's appendices (https://static-content.springer.com/esm/art%3A10.1038%2Fs41467-020-19045-9/MediaObjects/41467_2020_19045_MOESM1_ESM.pdf). The *Journal of Proteome Research* celebrated the achievement by publishing in October a Virtual Issue and Editorial[3] dedicated to the HPP and the human proteome draft. This support continues in December 2020 with the publishing of the eighth annual Special Issue of the *Journal of Proteome Research* on the HPP. Even with many HPP research laboratory closures during the lockdown in many countries for many months to lessen the spread of SARS-CoV-2 to the many, remarkably, this Special Issue publishes 15 major contributions from the two HPP initiatives, the Chromosome-Centric HPP (C-HPP)[4,5] and the Biology/Disease (B/D-HPP) HPP,[6] and the four Resource Pillars of the HPP, Mass Spectrometry, Antibody Resource, Knowledgebase, and Pathology.

LIGHTING UP THE HUMAN PROTEOME

The Special Issue commences with the annual HPP Metrics paper by Omenn et al. The neXtProt[7,8] database (https://www.nextprot.org) released the landmark version of the human proteome on January 17, 2020 following the identification of >90% of the human proteome by curating and compiling peptide and protein identification data from PeptideAtlas[9] (http://www.peptideatlas.org) and MassIVE (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp). Whereas the *Nature Communications* Decal Report describes the history and legacies of the HPP and the progress made in identifying the human proteome over the past 10 years to reach the 90% coverage milestone, the HPP Metrics paper provides fine-grain detail of the progress and challenges in credibly identifying the human proteome over the past year. These two significant HPP papers report the identification of 17 874 proteins classified as protein existence level 1 (PE1) proteins translated from the human genome, which today is calculated to contain 19 773 protein-coding genes. This represents 90.4% of the human proteome now identified at the protein level to high stringency and according to HPP Guidelines.[10]

What have we learned of the human proteome and the HPP progress over the past year? Proteins are the fabric of life. Our genomes just provide the overall instruction set on which protein "threads" are used and when our proteins are woven into the fabric. However, it is our proteins and their interactions that determine the nuanced details of the weave and knots that form the amazing complex tapestry pattern that defines humans. The intricate tapestry of what makes a human a human starts with the genetic blueprint and was once thought to end with our proteins. We now know better. Genomic and, as important, proteomic variability defines our individuality. Bespoke proteoforms of each protein, in turn, generate human diversity, individuality, and responses to different diseases, aging, and other stressors. How the fabric of this tapestry develops and grows to full size and then changes over time, day to day and over a lifetime, in sickness and in health, must be understood. Because genomics cannot do this, proteomics is the start point. Genomics also cannot provide all of the answers or diagnostics for diseases lacking a genetic basis. Critically, genomics cannot provide information on disease activity status and on-target drug activity. Accurate diagnosis for most diseases will come not from mere measuring of the parts—we all have them—but from their proteoforms present, their post-translational modifications (PTMs), and the interactions that determine the cell and tissue status, or disease stage.

THE DARK SIDE OF THE PROTEOME

"There is no dark side of the moon, really. Matter of fact...",[11] just a far side that remained unseen by humans until Apollo 8. The dark proteome, representing unseen or missing proteins lacking evidence of their existence at the protein levels (PE2−PE4 proteins), now comprises just 10% of the human

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proteome. This remains hidden as "the dark side" and contains 1899 proteins. The gibbous moon featured on the cover of this Special Issue of the Journal alludes to the 90 and 10%. The task of uncovering the missing proteins is becoming exponentially more difficult. As described in detail in the HPP Metrics paper, in the past year, just 180 proteins have been newly promoted to PE1 status, with missing protein numbers reduced by 230. The Chromosome X team (Japan) has enjoyed great success in identifying missing proteins, with 35 now PE1 proteins in neXtProt from 2019 alone. Nonetheless, a larger number of missing proteins that were reported "found" and were discussed in the seventh annual Special Issue of the *Journal of Proteome Research* on the HPP in 2019 failed to satisfy the HPP Guidelines 3.0, were not captured by PeptideAtlas for reanalysis, or failed reanalysis and so were not promoted to PE1 status. These remain only as candidate "found" missing proteins. As detailed in table 2 of the Metrics paper, the identification of missing proteins on a chromosome-by-chromosome basis by the international C-HPP teams during 2020 reveals the progress made, especially for chromosomes 1 (China) and 19 (Mexico). But success is hard and often elusive. Employing a well-established 2D fractionation methodology concatenated with SRM assay readouts, Vavilov et al. (Russia) were unable to obtain full coverage of chromosome 18 proteins present in HepG2 samples despite mRNA evidence of their expression. This again emphasizes the increasing difficulty in detecting PE2−4 proteins in conventional cell samples by conventional approaches, highlighting the need to seek less commonly analyzed cells and to utilize peptide spectral enhancement technologies such as spiked-in isotopically labeled synthetic peptides, as proposed by Vavilov and colleagues and successfully employed by Kotol et al. for drug targets and cytokines in serum.

Missing proteins can be simply missing if they lack features rendering them amenable to mass-spectrometric detection or, more often, if they are limited in abundance, are expressed by rare cells, or are in cells that are rarely analyzed, are at rare times in human development, or are in rare host responses to now rare health challenges, which in today's world are thankfully only remembered by history. Addressing this, Zhang et al. levered a metastatic cell line derived from brain cancer, D283 Med, to find and validate 12 missing proteins. Alikhani et al. and the Persian Y Chromosome team's Perspective paper suggests that CRISPR engineering and organoid technologies of human pluripotent stem cells establish a promising platform for missing protein identification and the functional characterization of human proteins lacking known functions.

From the Human Protein Atlas and the Antibody Resource Pillar comes the remarkable achievement of the "enhanced validation" of nearly 6000 antibodies directed toward 3775 proteins in many tissues detailed by Sivertsson et al. (Sweden). This led to the localization of 56 candidate missing proteins and 171 uncharacterized PE1 (uPE1) proteins lacking any known function. The power of specific and well-characterized antibodies in finding missing proteins and aiding in the functionalization of proteins has been long recognized. This study on such scale shows its true potential. The authors propose that correlating immunohistochemistry using enhanced antibodies, single-cell transcriptomics, and mass spectrometry analyses of the same sample will develop knowledge on the epitopes and the types of protein affected by immunohistochemical sample preparation and the potential of the enhanced validation of antibodies to help identify and locate more confidently missing proteins. Nonetheless, this again raises the need for community-accepted guidelines for the use of antibody evidence of PE1 classification to the same stringency as required for mass spectrometry.

DARK FUNCTIONS

In addition to finding missing proteins, an additional ∼1937 dark proteins with unknown function—including 1254 uPE1 proteins,[12,13] representing a more experimentally tractable set of proteins than the uncharacterized and also missing proteins (uMPs)—and proteins with no known structure[14,15] pose steep challenges ahead for the HPP community in shedding light on the dark proteome. Lachén-Montes et al. and the Spanish team (Chromosome 16) identified a host of dark proteins in synaptic terminals, one of which, PITH domain-containing protein 1 (PITHD1), in the olfactory bulb, was further characterized for its function in mice, which were employed as a tractable model. By association evidence, in human Alzheimer's disease, PITHD1 is specifically increased in abundance, providing indirect insight into the function of this protein. At a different level, Na et al. (Korea) exposed subtleties in unknown functions by uncovering a novel role implicit in retarding hepatocellular carcinoma cell proliferation by human carboxylesterase 1, in particular, the singular importance of N-linked glycosylation at Asn79.

Bioinformatics approaches developed, as described in this Special Issue, to tackle the uPE1 challenge include a guilt-by-association bioinformatics approach from the Spanish team in the paper by González-Gomariz et al. In an innovative approach, the authors employed web search tools, such as the Google PageRank algorithm, to develop UPEFinder. This new tool maps the similar topography between selected data sets, including the Gene Ontology, DISEASE, and Molecular Signatures databases, to access functionally relevant information so as to bioinformatically suggest candidate biological functions for uPE1 proteins that can be tested. Hwang et al. and the Korean Chromosome 11 team used the successful I-TASSER/COFACTOR approach[16] to predict 2413 GO terms for 22 uPE1 chromosome 11 proteins. By further winnowing of GO terms using the new algorithms the authors describe, three proteins were identified for further promising functional study.

The Chromosome 2 and 14 teams from Switzerland and France, respectively, have a long history of coming together in analyses of male reproductive tract proteomes that has aroused considerable interest. These sensitive tissues have proven to be a rising source of missing proteins, as detailed in several Special Issue papers over the past years.[17] Enlarging this work, Vandenbrouck et al. tackled the uPE1 neXt-CP50 Challenge in a cohort of 421 uPE1 proteins found in higher abundance in the male reproductive tract by the compilation of diverse evidence. To functionally annotate such proteins, contextual information from the literature, protein−protein interactions, expression levels, and cellular localization were employed in a knowledge-driven approach to suggest rational, knowledge-founded hypotheses that can be experimentally tested in a targeted manner with a higher probability of precise results and fewer false starts. This approach represents a welcome departure from phenotypic screens at scale conducted in a knowledge-agnostic manner, attempting to deorphanize the protein function. The testis was also the target of an extensive multiprotease proteomic analysis by Wu et al. (China), who identified and validated four new missing protein candidates

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and a large number of unvalidated candidates. In other work from the B/D-HPP teams, Choi et al. (Qatar) provided quantitative proteome information on adipocytes that will prove useful for understanding adipocyte differentiation and lipid metabolism.

ENLIGHTENED POST-TRANSLATIONAL MODIFICATION ANALYSES

Tackling and making sense of the incredible complexity of the human proteome is a major focus of the HPP. Proteome complexity arises in part from the >400 PTMs of amino acids[18] and the different lengths and compositions of proteins arising from splice variants, alternate start sites, and the proteolytic processing of every protein in the proteome,[19] which together generate millions of proteoforms. Moreover, the myriad of protein−protein interactions needs to be understood as well as the dynamics of protein complexes, which change for ∼50% of proteins in step with different states of the cell. Proteomics is essential to the understanding of human health and disease, as genomics alone cannot quantify PTMs in a protein, which can turn an activity on or off and change a protein's localization, turnover, and interactions with other proteins.

The correct identification of the actual PTM site is critically important yet often technically or bioinformatically challenging. Hoopmann et al. (USA/Canada) tackled this in the "phospohopeptide challenge" with the MS Resource Pillar of the HPP. Standardized sets of 94 phosphopeptides were analyzed by 22 laboratories using different approaches, MS instrumentation, and bioinformatics. The data were reanalyzed in a consistent manner that pointed out the challenges of correct phosphopeptide site identification and the weaknesses in current software. Performance analysis using the diverse approaches of this community will prove useful to devise improved methods for phosphosite identification. The nature of the challenge itself also suggests a platform to assess and develop standards for other new techniques in the mass-spectrometry-based proteomic identification of PTM sites.

In infections, such as that from SARS-CoV-2, and diseases, for example, the ensuing COVID-19, missing proteins may be selectively expressed in host−pathogen interactions, but changes in the activity of neither the disease-relevant proteins nor the virus-binding partners can be predicted by genomics. Hence, the zoonotic origin of SARS-CoV-2 was explored by Huang et al. (USA), modeling the spike protein cell receptor, ACE2, interaction in 285 animal species. In addition to providing insight into the animal origin of SARS-CoV-2, which still remains elusive, the development of this modeling approach showed great potential in developing hypotheses for field testing.

This Special Issue submission deadline was too early for the many COVID-19 studies expected soon, but, again, the importance of identifying changes in PTMs and using proteomics for accurate early diagnosis cannot be stressed enough. The life-threatening cytokine storms in severe COVID-19 cases represent a massive disruption of the proinflammatory cytokine and chemokine levels and their activity that normally are precisely regulated in expression over time, cell/tissue location, and bioactivity. Most chemokines that control leukocyte cell migration and activation are modified by PTMs such as citrullination, which converts arginine to the amino acid citrulline, or by precise proteolytic processing that may remove one or a handful of amino acids.[20] This occurs during the active disease phase to activate, then inactivate chemokines and even to switch cell surface receptors to lead to a totally different signal in the target cells. Cleavage can also generate antagonists that prevent further signals from being transduced in the cellular pathway. But all of these cytokine proteoforms are generated from exactly the same gene. Hence, knowing that information at the transcript level alone does not resolve the temporal expression of the all-important bioactivity status of these cytokine proteoforms in the patient. Therefore, proteomics urgently needs to be deployed to understand such devastating storms and the pathobiology of COVID-19.

Relevant to monitoring cytokine activity as a potential biomarker of the disease stage in infections like COVID-19 and other diseases is the work of Kotol et al. The Swedish group used information derived from the Human Protein Atlas to devise a series of isotopically labeled peptides with corresponding PRM assays for the detection of 21 drug targets and biomarkers in human plasma. By sampling normal subjects' plasma at four time points over a 1 year time frame to generate longitudinal data on the relative levels and changes of the 21 targets, the proteins' potential as a constellation of multiplex biomarkers was assessed. Using crucial activity-affecting features or PTM proteoforms of such proteins as biomarkers can provide accurate, timely information on the active disease activity status or the on-target drug activity that patients experience. This is vital information to inform diagnosis, treatments, and patient management. Thus proteomics really holds the key to devising new accurate diagnostic tests for personalized medicine. These analytical approaches can then be translated as more simple ELISAs and other tests suitable for deployment in hospitals and diagnostic laboratories or, as shown feasible by Kotol et al., by multiplexed targeted proteomics, providing more nuanced information that can be key to accurate, early, and appropriate medical decision making.

In shedding light on the human proteome and the other advances reported in this Special Issue of the *Journal of Proteome Research* on the HPP, there is success and inspiration in a year of darkness and devastating losses for many. Proteomics will contribute much to understanding the pathobiology of COVID-19 and monitoring vaccine and on target new treatment efficacy in the months ahead. Relevant to the HPP, I predict that evidence of missing proteins will be found if sought in the proteomes of SARS-CoV-2-infected cells as new infection responder proteins.

Finally, the Journal welcomes the new Chair of the HPP, Dr. Rob Moritz, Institute for Systems Biology, Seattle, as a Guest Editor for the ninth annual Special Issue of the *Journal of Proteome Research* on the HPP in 2021. The Guest Editorial team that was responsible for this eighth Special Issue, comprising Drs. Young-Ki Paik, Gil Omenn, Lydie Lane, Eric Deutsch, and Fernando Corrales and myself, wish you and your loved ones health and safety during the dark days of the pandemic that are still ahead until science delivers vaccines and new treatments and, hopefully, a new understanding of and appreciation for the value of science to the world.

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Notes

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