REVIEW

Cell Biology

Proteomics
www.proteomics-journal.com

Phenotypic Analysis of Organoids by Proteomics

*Alexis Gonneaud, Claude Asselin, François Boudreau, and François-Michel Boisvert**

The development of 3D cell cultures into self-organizing organ-like structures named organoids provides a model that better reflects in vivo organ physiology and their functional properties. Organoids have been established from several organs, such as the intestine, prostate, brain, liver, kidney and pancreas. With recent advances in high-throughput and -omics profiling technologies, it is now possible to study the mechanisms of cellular organisation at the systems level. It is therefore not surprising that these methods are now used to characterize organoids at the transcriptomic, proteomic, chromatin state and transcription factor DNA-binding levels. These approaches can therefore provide a wealth of information regarding both the mechanisms involved in different diseases, and those involved in cell responses to different conditions, in a more in vivo setting. The authors provide an overview of the potential applications of quantitative mass spectrometry with organoid culture, and how the use of large-scale proteome measurements is emerging in different organoid systems.

1. Introduction

Since the first proteome characterization,[1] mass spectrometry has allowed the simultaneous identification and quantification of large numbers of proteins in cell and organ samples. Determining the cellular proteome allows the establishment of a functional map of protein expression patterns beyond gene expression, and as such is complementary to genomics studies. The properties assessed by proteomic studies include protein abundance, protein–protein interactions, post-translational modifications, subcellular localization and protein turnover. This provides a dynamic overview of the changes that occur during different biological processes [2]. Until recently, studies have focused on the proteomic analysis of 2D cell lines in relation to different pathologies, such as cancer.[3–5]

Cell culture models display several advantages to study the effect of pharmacological drugs, such as anti-cancer products. From a biological point of view, traditional 2D cell culture models are rapid and practical, and allow a first evaluation of the effects related to drug treatments. However, homogeneous cell culture models are far removed from the normal biological context. Indeed, cell-cell interactions and cell-matrix contacts are frequently altered. Tissue structure and complexity, as assessed by the simplified multicellular composition of these models, are missing. One way to address this oversimplification is to develop spheroid 3D models. These models are closer to in vivo models, without the inconvenience of time and associated costs, and can be established from previously characterized cell lines. This more complex system implies cells folding into spheres,[6] coupled to different microenvironments or cell co-cultures.[7] Cell–cell and cell–matrix interaction models [8] are more relevant and closer to normal and pathological biological processes,[9] and allow the impact evaluation of the tumor stroma.[10] Spheroids originating from cancer cells are characterized by different growth zones, either hypoxic or proliferative, common tumor-related phenotypes. While 2D cell cultures are mostly in a proliferative stage, 3D spheroids display different proliferative stages .[11] 3D spheroid culture models are thus more complex, thereby providing improved drug discovery assays in order to better evaluate and measure drug-dependent effects on cell polarization, survival or differentiation, among others.[12] Effective pharmacological doses may be determined by measuring the penetration of the agent into spheroids. For example, spheroids obtained from HCT116 human carcinoma colon cells were treated with an anticancer drug, irinotecan. Irinotecan metabolites were detected by MS/MS and MALDI IMS, and irinotecan distribution in spheroids was reconstructed, according to drug exposure time and concentration [13]. Comparison between 2D cell models and 3D spheroid cultures from different cell lines reveals extensive differences, notably in cell interactions with the environment through adhesion molecules, among others.[14,15]

In 2009, an intestinal organoid cell culture model was developed. Lgr5-expressing intestinal epithelial cell stem cells, grown in Matrigel and in a specific growth and differentiation medium, were able to rebuild an intestinal-like structure referred as "mini-gut".[16] This structure was composed of a proliferative crypt/bud compartment and a differentiated center area surrounding a lumen and containing differentiated intestinal epithelial cells, including Paneth cells, goblet cells and enterocytes.[16] Paneth cells were located between stem cells, thus providing factors needed for stem cell growth and maintenance, including Wnt3, a β-catenin proliferative pathway activator.[17] Since then, significant progress has been made in defining optimal conditions to allow growth, expansion and differentiation of stem cells from several different tissues.[18–22] Indeed, organoids have been cultured and maintained from various tissues, including stomach,[23]

A. Gonneaud, Prof. C. Asselin, Prof. F. Boudreau, Prof. F.-M. Boisvert
Department of Anatomy and Cell Biology
Université de Sherbrooke
Sherbrooke, Québec, Canada
E-mail: Francois.Michel.Boisvert@USherbrooke.ca

DOI: 10.1002/pmic.201700023

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

brain,[24] lung [25] and liver .[26] Thus, organoids better reflect tissue characteristics in both normal and diseased physiological conditions, as opposed to 2D cells or 3D spheroid models.[27,28] Unlike cancer cell lines, organoids maintain all the variables specific to the original epithelial cell, including the tumor cell.[29] As a result, organoid culture is becoming an attractive model for personalized medicine, as it allows the testing of existing and experimental treatments on samples with distinct genomic individual signatures.[30,31] Organoids offer the opportunity to study, in a "body-like" manner, the protein and gene expression variations occurring in different pathologies or environmental conditions [32]. The ability to culture these mini-organs raises the question of their use for quantitative proteomics, in order to accurately quantify proteins in physiologically relevant systems. Quantitative proteomics allow the measurement of proteome changes during cellular differentiation, or in response to different inhibitors and drugs. With the recent promise of adapting organoid technology for precision medicine,[33] the combination of organoid culture with quantitative proteomics will become advantageous to quantify global changes in protein expression, thereby identifying novel signalling pathways and targets upon defined environmental contexts (**Figure 1**).

In this review, we discuss recent advances in proteomics study approaches with organoid models. We discuss and compare different quantitative proteomics methods that have been used, and we highlight results of some studies published so far, underlining the importance of this emerging area of research.

2. Quantitative Proteomics Approaches to Study Organoids

A number of mass spectrometry-based quantitative approaches have been developed to measure protein changes in several samples under different conditions. A common feature for quantitative proteomics methods is to use strategies to generate small differences in peptide mass, in order to quantify samples analyzed simultaneously by the mass spectrometer. While several methods exist, so far only a few methods have been used for quantifying proteome differences in organoids[34–37] (**Figure 2**).

Stable isotope labeling with amino acids in cell culture, or SILAC, uses stable and non-radioactive isotopic atoms to generate a small difference in peptide mass that is then quantified by mass spectrometry analysis.[38,39] SILAC allows quantitative protein analyses by comparing the mass of light and heavier forms of a given peptide from each differentially labeled protein, resulting from the presence of heavier and stable isotopes such as ^{13}C, ^{2}H and ^{15}N. Cells are metabolically labelled in specialized growth media in which specific amino acids, typically arginine and lysine, are replaced with amino acids containing the corresponding heavy isotope-substituted carbon, hydrogen and/or nitrogen forms.[40] After stable isotope incorporation of proteins in cellulo, proteins cleaved at the substituted arginine or lysine by trypsin generate peptides with a shift in mass relative to the control unsubstituted peptide, and this can be resolved by mass spectrometry. The intensity ratios between "light" and "heavy"

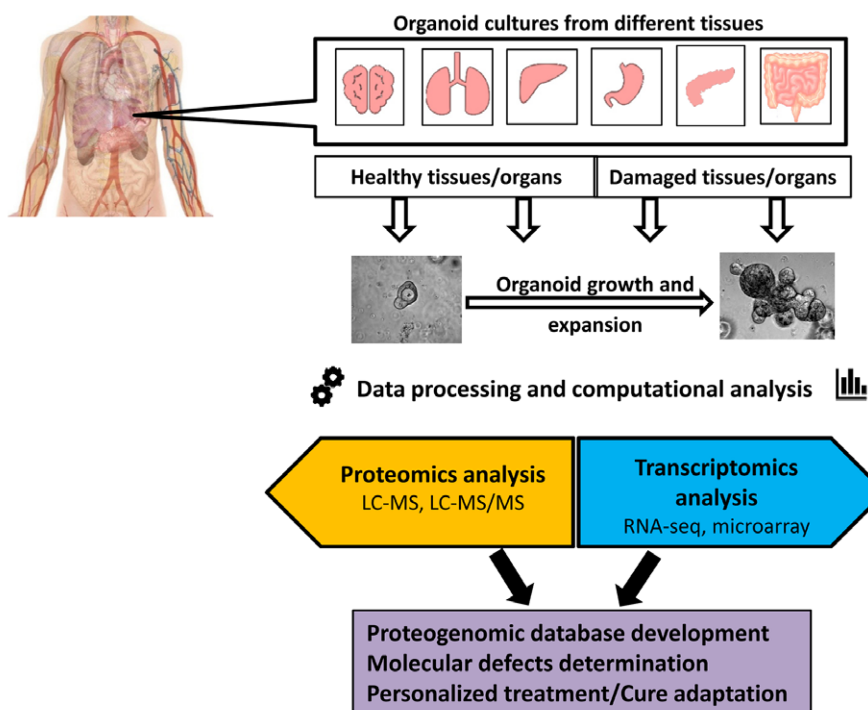

Figure 1. Organoid culture and therapeutic potential.
Since their first establishment from the gut in 2009, organoids are now generated from multiple tissues, including brain, lung, liver, stomach, pancreas or intestine. Organoid culture provides a model that closely recapitulates the in vivo organization, functions and responses of the tissue of origin. Organoids can be cultured and amplified prior to analysis with different -omics approaches, notably proteomic assays and RNA-sequencing. Comparing organoids derived from healthy and abnormal tissues allows multiple applications for disease modeling of different pathologies, such as cancer and inflammation. It thus provides an attractive model to define molecular defects in patients and to adapt treatments.

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

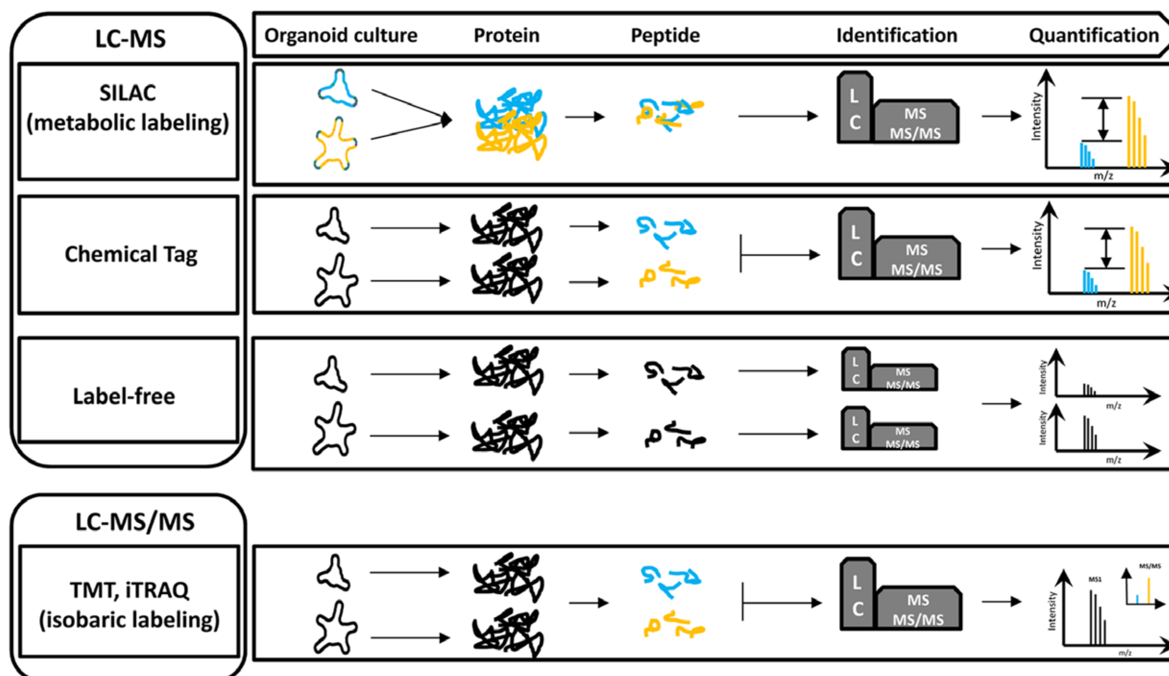

Figure 2. Comparison of available proteomics technologies for organoid analysis.
Many novel proteomic approaches have now been applied to study organoids. A common feature for quantitative proteomic methods is to use strategies to generate small differences in peptide mass, in order to quantify samples analyzed simultaneously by the mass spectrometer. These methods use either metabolic labeling (SILAC), addition of a tag through chemical reactions for the quantification at the MS level (chemical tag), or addition of an isobaric tag for quantification at the MS/MS level (isobaric labeling). Alternatively, quantification can be based on the intensities of peptides (label-free quantification).

peptide signals identified by mass spectrometry directly correlate with the relative amount of cognate proteins from each sample. This method has been extremely successful for quantitative analysis of cell and organelle proteomes and has become particularly attractive for comparative studies of protein localization, modifications and interactions.[41]

A different approach for the quantification that also uses a shift in mass measured at the MS level can be done following enzymatic digestion of proteins, and then labeling the resulting peptides using labels of different masses, with the possibility of adding an affinity tag to further purify the labeled peptides.[42] For example, peptides obtained from human colon organoids were tagged by stable isotope dimethyl labeling.[36,43] The advantage of this method is that it greatly reduces the time necessary for in vivo incorporation of isotopes and does not require the preparation of complex media in comparison to SILAC, but it does introduce variations due to differences in preparation from mixing the samples at later steps.

Isobaric labeling (Tandem Mass Tag, TMT; isobaric Tags for Relative and Absolute Quantitation, iTRAQ) uses various isobaric chemical groups with the same mass, but with different fragmentation properties during tandem mass spectrometry. This allows the measurement of reporter ions with different masses. These tags generally contain an amine-reactive group that undergoes N-hydroxysuccinimide (NHS) reactions with peptides after trypsin digestion, and independently labeled samples are then mixed in equal ratios. During mass spectrometry analysis, fragmented peptides yield the peptide sequence as well as the reporter tags, whose intensities are measured to quantify the relative ratio of the peptide, and thus the protein, within the combined samples. Because these tags have all the same mass and are measured at the MS/MS level instead of the MS level such as in the previous methods, the labelled peptides are indistinguishable during the chromatography and single MS mode. This allows a greater number of different samples to be compared.

Multiple reaction monitoring (MRM) is a method that targets specific peptides in complex samples by selecting predefined peptide ions and a combination of specific fragment ions for accurate quantitation.[44] In contrast to the other quantitative methods, MRM allows absolute quantitation by using isotopically labeled synthetic peptide internal standards, which are designed to be identical to target peptides.[45] Known concentrations of heavy synthetic peptides are added to the sample, and the concentration of the target protein can be calculated by measuring the observed peptide against the internal heavy standard.[44] MRM therefore holds great potential for the validation of candidate proteins in quantitative studies, but requires previously identified protein targets. So far, MRM quantitative proteomics methods have not been used in organoid model studies.

3. Strengths and Limitations of the Different Quantitative Approaches

An important difference between these methods is that isobaric labeling or post-enzymatic labeling does not require cell culture

 © 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

in specialized isotopic amino acid media, and can therefore tag samples taken directly from patients or animals. Nonetheless, prolonged organoid culture is required to obtain sufficient material as well as for organoid proliferation and differentiation prior to sample preparation for mass spectrometry, minimizing the difference of the additional time for growth needed to insure sufficient SILAC organoid labeling. Organoids, either directly or following isotope incorporation, can be frozen for storage, preservation and future assays, which shortens the time of culture after the initial incorporation.[35] Thus, culture time differences may be minimized since SILAC-labeled frozen organoids can be used to restart organoid growth.[35] As opposed to isobaric labeling methods, SILAC is more limited in the number of combinations available for isotope labeling because of the number of labels that can be simultaneously used. Indeed, since peptide quantification for SILAC is performed at the MS level, an increased number of labeling conditions results in a decrease in signal intensity. Thus, the major advantage of iTRAQ or TMT is the possibility of multiplexing up to ten or more different samples in one analysis. On the other hand, the strength of SILAC is that as samples are combined prior to cell lysis and trypsin digestion, errors following extensive sample manipulation and preparation prior to labeling are prevented, resulting in a more accurate quantification.

Label-free quantification is generally based on the precursor ion intensity and is measured at the MS1 level. The total ion intensity of the peptide signal, considering all isotopic peaks, is integrated, and used as a quantitative measurement of the original peptide concentration. In contrast to using isotopes or tags, every sample needs to be measured separately, and peptide signals are compared between multiple LC-MS runs using elution time as well as their mass over charge ratio. Several variations of peptide quantification, as well as computational and statistical analysis, have been developed.[46] Because label-free quantification does not rely on any additional labeling or sample preparation, it is applicable to any kind of sample and has no limits in the number of samples that can be compared. However, accurate and reproducible proteome-wide quantification remains a challenge, notably in the detection of small changes between biological samples.

MRM is particularly suitable for low abundance peptides and provides absolute quantification compared to other methods.[47] However, absolute quantitation, which requires suitable internal standards that needs to be synthesized and optimized for each target peptide, only measures the abundance of individual peptides, not the whole protein. Therefore, the number of proteins that can be quantified using this approach is rather limited. MRM thus remains an excellent method of choice for the quantification of specific proteins, but is less amenable for discovery-based studies relying on identifying and quantifying large number of proteins that have not been previously selected.

Mass spectrometry-based proteomics approaches can be applied to study not only protein abundance, but also to measure post-translational modifications, protein subcellular localization and interaction networks. The major challenge is the need for sufficient amount of starting material for biochemical enrichments, since the resulting purified fraction is in the order of 1–5% of the starting material. Current proteomics studies have used starting material in the range of 30–200 μg of total organoid-derived proteins for comparative protein expression analysis. Considering that most applications, such as phosphoproteomics and affinity-purification of protein complexes, require more than one milligram of proteins, such experiments necessitate the scaling up of organoid production. This is certainly within the range of cultures currently done, with a need of an approximately five-fold increase in the amount of organoids.

4. Proteomics on Organoids

The first report using quantitative mass spectrometry to study organoids was published in 2015 by Boj et al.[37] Gene expression patterns from normal or ductal pancreatic murine cancer were analysed by RNA-seq. iTRAQ labeled proteins were obtained from murine pre-invasive pancreatic intraepithelial neoplasm organoids (mP), multiple primary tumor organoids derived from Kras$^{+/LSL-G12D}$, Pdx1-Cre and Kras$^{+/LSL-G12D}$, Trp53$^{+/LSL-R172H}$, Pdx-Cre mice (mT), as well as normal pancreatic ductal organoids (mN). Their proteomic analysis identified approximately 710 proteins increased or decreased between mP and mN organoids, and 1047 proteins up- or down-regulated between mT and mN organoids. However, a comparison with RNA-seq data revealed only a few protein changes correlating with transcriptional changes. A comparison of molecular pathways differing in both -omics data uncovered differences in anabolic and catabolic pathways, including perturbations in fatty acid metabolism, between mP and mT organoids. This correlated with pancreatic ductal adenocarcinoma progression. Thus, analysis of pancreatic organoids by proteomics approaches could be a strong alternative for characterizing human pancreatic cancer, as biopsies can be cultured in organoids for rapid evaluation and selection of appropriate treatments.

In 2016, Williams et al. [34] have determined the effect of environmental chemical stresses on 8plex iTRAQ labeled proteins from murine mammary organoids. Organoids were treated with physiologically relevant concentrations of xenobiotic agents, such as bisphenol A, mono-n-butyl phthalate and polychlorinated biphenyl 153. Control and xenobiotic-treated samples were randomized in four groups, before analysis of different protein sample comparisons. This led to the identification and quantification of approximately 4000 proteins. Xenobiotic treatments induced mammary organoid morphology modifications, correlating with protein changes associated with some gene ontology-identified cellular processes, including proliferation, cell cycle and translation. Analysis of the proteomic data revealed modifications of several alternatively spliced proteins, suggesting that environment stress-induced disturbance could impact mammary development, and, ultimately, breast cancer initiation or progression.

Gonneaud et al. [35] have established intestinal jejunal organoid growth conditions for SILAC incorporation, including the optimal time for efficient isotope incorporation, and the possibility to freeze SILAC-labeled organoids for future use. Treatment with the class I HDAC inhibitor CI994 induced morphological changes, resulting in smaller and less differentiated organoids characterized by an apparent reduction in intestinal epithelial crypt formation. The proteomic analysis identified and quantified over 2500 proteins in these purified organoids allowing protein comparison, as well as evaluation of the reproducibility using technical and biological replicates. The results showed

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

increases in absorptive lineage markers, in contrast to decreases in secretory lineage proteins, consistent with the observed phenotype. Additional gene ontology analysis of differential protein expression patterns in response to the inhibitor revealed increased metabolic-related processes and decreased proliferation-associated functions related to DNA replication, cell cycle or RNA processing.

Another investigation combining -omics approaches was published by Cristobal et al. [36]. Organoids derived from seven different patients with colorectal cancers were used to generate personalized tumor profiles. Proteins from organoids were labeled with a quantitative dimethyl isotope labeling method, and gene transcript profiling was assessed by microarray analysis. Of 5,790 proteins identified, about 5% of these proteins were considered differentially expressed between tumor-derived and control organoids. Of those, 78 proteins were increased while 227 proteins were decreased. Protein data analysis established a specific signature for each patient-derived organoid, allowing a classification of the different types of colorectal cancer. For example, the DNA mismatch repair protein MSH3 was downregulated in only one patient, but correlated with an MSH3 frameshift deletion in that patient, confirming the identification of specific proteins affected in some of the patients. However, only 22 proteins out of 305 displayed concordant proteome and transcriptome changes. This study underlines the potential of patient-derived organoids for personalized disease characterization.[36].

In another study, Dakic et al.[48] have characterized the effects of a hallucinogenic molecule, 5-methoxy-N, N-dimethyltryptamines (5-MeO-DMT), on 45-day cultured cerebral organoids treated for 24 h. Proteome quantification was done with label-free protein samples. Of the 6728 proteins identified, 934 were differentially expressed following the treatment with 5-MeO-DMT. Pathway analysis revealed downregulation of NFAT and NF-κB signaling pathways in response to the drug, suggesting anti-inflammatory effects, as well as modifications in proteins related to microtubule and cytoskeletal organization, and dendritic spine formation. Cell death and neurodegeneration pathways were decreased, suggesting a protective function. Interestingly, this drug-dependent pattern of protein expression was only observed in the brain organoid model, but not in 2D neuronal cell cultures.

These studies highlight the potential of organoids as model systems for a variety of healthy and diseased tissues. Proteomics data will facilitate the mechanistic understanding of differences observed in organoids derived from patients with different genetic backgrounds, or in organoids treated with different drugs or signals. Until recently, it was generally accepted that there would be a proportional relationship between cell and tissue mRNA and protein expression levels. However, several studies comparing mRNA and protein expression data from cells under similar conditions have not shown a high correlation between both, indicating numerous additional processes regulating protein amounts within a cell as opposed to mRNA levels, such as translation regulation and protein stability.[49] Nonetheless, combined transcriptome and proteome analysis can provide further insights into mechanisms and biological pathways responsible for differences in protein expression.

5. Biological Perspectives

Organoids are fast becoming a promising model to study cells within a context closer in resemblance to normal tissue conditions. Thus, these mini-organs can better reflect tissue organisation as well as complex diseases, and patient-derived cells can be used to characterize the environment as well as to measure more accurately the effect of drug therapies.

Initially, most -omics data on organoids were focused on transcriptomic analyses.[23,50–52] These genetic approaches allow the study of the genome, the identification of mutations and characterization of gene transcript expression as well as their variants (alternative splicing). The information at the protein level is another dimension that allows to quantitatively observe changes in functional pathways and biological processes with high accuracy and sensitivity. Using innovative proteomics methods, it is possible to access information not only at the protein level, but also to measure post-translational modifications, protein localization and interaction networks. The study by mass spectrometry using different quantitative techniques can identify the proteome of different types of organoids and how they react to different conditions. Organoid can be generated from samples taken directly from patients, then expanded, harvested and analyzed by mass spectrometry.[36] Moreover, the generated organoids can be used to evaluate which therapeutic approaches is most effective in this context and can also be stored for future testing.

Acknowledgements

F.M.B funding: Natural Sciences and Engineering Research Council of Canada (grant number 418404 to FMB). F.B. funding: Natural Sciences and Engineering Research Council of Canada (grant number 262094 to F.B.). C.A. funding: Crohn and Colitis Canada. F.M.B., F.B. and C.A. are members of the FRQS-funded “Centre de Recherche du CHUS”.

Conflict of Interest

The authors have declared no conflict of interest.

Keywords

3D cell culture, Mass spectrometry, Organoids, Quantitative proteomics, SILAC, Stem cells

Received: April 3, 2017
Revised: May 24, 2017

[1] P. James, *Q. Rev. Biophys.* **1997**, *30*, 279.
[2] M. Larance, E. Pourkarimi, B. Wang, A. Brenes Murillo, R. Kent, A. I. Lamond, A. Gartner, *Mol. Cell. Prot.* **2015**, *14*, 1989.
[3] A. Rubporn, C. Srisomsap, P. Subhasitanont, D. Chokchaichamnankit, K. Chiablaem, J. Svasti, P. Sangvanich, *Cancer Genom. Proteom.* **2009**, *6*, 229.
[4] S. Tanaka, A. Sakai, K. Kimura, H. Yoshida, H. Fushitani, A. Ogata, A. Miyamoto, M. Fukushima, A. Wada, N. Tanigawa, *Int. J. Oncol.* **2008**, *33*, 361.
[5] S. J. Pitteri, L. JeBailey, V. M. Faca, J. D. Thorpe, M. A. Silva, R. C. Ireton, M. B. Horton, H. Wang, L. C. Pruitt, Q. Zhang, K. H.

 © 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Cheng, N. Urban, S. M. Hanash, D. M. Dinulescu, *PLoS One* **2009**, *4*, e7916.
[6] S. L. Ham, R. Joshi, P. S. Thakuri, H. Tavana, *Exp. Biol. Med.* **2016**, *241*, 939.
[7] H. Jaganathan, J. Gage, F. Leonard, S. Srinivasan, G. R. Souza, B. Dave, B. Godin, *Scientific Reports* **2014**, *4*, 6468.
[8] E. Cukierman, R. Pankov, D. R. Stevens, K. M. Yamada, *Science* **2001**, *294*, 1708.
[9] M. W. Tibbitt, K. S. Anseth, *Biotechnol. Bioeng.* **2009**, *103*, 655.
[10] S. Talukdar, M. Mandal, D. W. Hutmacher, P. J. Russell, C. Soekmadji, S. C. Kundu, *Biomaterials.* **2011**, *32*, 2149.
[11] A. C. Luca, S. Mersch, R. Deenen, S. Schmidt, I. Messner, K. L. Schafer, S. E. Baldus, W. Huckenbeck, R. P. Piekorz, W. T. Knoefel, A. Krieg, N. H. Stoecklein, *PLoS One* **2013**, *8*, e59689.
[12] R. Edmondson, J. J. Broglie, A. F. Adcock, L. Yang, *Assay Drug Dev. Technol.* **2014**, *12*, 207.
[13] X. Liu, E. M. Weaver, A. B. Hummon, *Anal. Chem.* **2013**, *85*, 6295.
[14] X. Yue, Lukowski, J. K. Weaver, E. M. Skube, S. B. Hummon, AB, *J. Proteome Res.* **2016**, *15*, 4265.
[15] S. A. Konze, L. van Diepen, A. Schroder, R. Olmer, H. Moller, A. Pich, R. Weissmann, A. W. Kuss, R. Zweigerdt, F. F. Buettner, *Mol. Cell. Prot.* **2014**, *13*, 990.
[16] T. Sato, R. G. Vries, H. J. Snippert, M. van de Wetering, N. Barker, D. E. Stange, J. H. van Es, A. Abo, P. Kujala, P. J. Peters, H. Clevers, *Nature* **2009**, *459*, 262.
[17] T. Sato, J. H. van Es, H. J. Snippert, D. E. Stange, R. G. Vries, M. van den Born, N. Barker, N. F. Shroyer, M. van de Wetering, H. Clevers, *Nature* **2011**, *469*, 415.
[18] X. Yin, H. F. Farin, J. H. van Es, H. Clevers, R. Langer, J. M. Karp, *Nat. Methods* **2014**, *11*, 106.
[19] T. Sato, D. E. Stange, M. Ferrante, R. G. Vries, J. H. Van Es, S. Van den Brink, W. J. Van Houdt, A. Pronk, J. Van Gorp, P. D. Siersema, H. Clevers, *Gastroenterology* **2011**, *141*, 1762.
[20] K. W. McCracken, E. M. Cata, C. M. Crawford, K. L. Sinagoga, M. Schumacher, B. E. Rockich, Y. H. Tsai, C. N. Mayhew, J. R. Spence, Y. Zavros, J. M. Wells, *Nature* **2014**, *516*, 400.
[21] A. Fatehullah, S. H. Tan, N. Barker, *Nat. Cell Biol.* **2016**, *18*, 246.
[22] B. R. Dye, D. R. Hill, M. A. Ferguson, Y. H. Tsai, M. S. Nagy, R. Dyal, J. M. Wells, C. N. Mayhew, R. Nattiv, O. D. Klein, E. S. White, G. H. Deutsch, J. R. Spence, *eLife* **2015**, *4*, e05098.
[23] S. Bartfeld, T. Bayram, M. van de Wetering, M. Huch, H. Begthel, P. Kujala, R. Vries, P. J. Peters, H. Clevers, *Gastroenterology* **2015**, *148*, 126 e126.
[24] M. A. Lancaster, M. Renner, C. A. Martin, D. Wenzel, L. S. Bicknell, M. E. Hurles, T. Homfray, J. M. Penninger, A. P. Jackson, J. A. Knoblich, *Nature* **2013**, *501*, 373.
[25] R. R. Nadkarni, S. Abed, J. S. Draper, *Biochem. Biophys. Res. Commun.* **2016**, *473*, 675.
[26] M. Huch, C. Dorrell, S. F. Boj, J. H. van Es, V. S. Li, M. van de Wetering, T. Sato, K. Hamer, N. Sasaki, M. J. Finegold, A. Haft, R. G. Vries, M. Grompe, H. Clevers, *Nature* **2013**, *494*, 247.
[27] P. H. Dedhia, N. Bertaux-Skeirik, Y. Zavros, J. R. Spence, *Gastroenterology* **2016**, *150*, 1098.
[28] H. Clevers, *Cell* **2016**, *165*, 1586.
[29] M. van de Wetering, H. E. Francies, J. M. Francis, G. Bounova, F. Iorio, A. Pronk, W. van Houdt, J. van Gorp, A. Taylor-Weiner, L. Kester, A. McLaren-Douglas, J. Blokker, S. Jaksani, S. Bartfeld, R. Volckman, P. van Sluis, V. S. Li, S. Seepo, C. Sekhar Pedamallu, K. Cibulskis, S. L. Carter, A. McKenna, M. S. Lawrence, L. Lichtenstein, C. Stewart, J. Koster, R. Versteeg, A. van Oudenaarden, J. Saez-Rodriguez, R. G. Vries, G. Getz, L. Wessels, M. R. Stratton, U. McDermott, M. Meyerson, M. J. Garnett, H. Clevers, *Cell* **2015**, *161*, 933.
[30] N. Barker, *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 19.
[31] R. E. Hynds, A. Giangreco, *Stem Cells* **2013**, *31*, 417.
[32] J. Drost, R. H. van Jaarsveld, B. Ponsioen, C. Zimberlin, R. van Boxtel, A. Buijs, N. Sachs, R. M. Overmeer, G. J. Offerhaus, H. Begthel, J. Korving, M. van de Wetering, G. Schwank, M. Logtenberg, E. Cuppen, H. J. Snippert, J. P. Medema, G. J. Kops, H. Clevers, *Nature* **2015**, *521*, 43.
[33] L. Huang, A. Holtzinger, I. Jagan, M. BeGora, I. Lohse, N. Ngai, C. Nostro, R. Wang, L. B. Muthuswamy, H. C. Crawford, C. Arrowsmith, S. E. Kalloger, D. J. Renouf, A. A. Connor, S. Cleary, D. F. Schaeffer, M. Roehrl, M. S. Tsao, S. Gallinger, G. Keller, S. K. Muthuswamy, *Nat. Med.* **2015**, *21*, 1364.
[34] K. E. Williams, G. A. Lemieux, M. E. Hassis, A. B. Olshen, S. J. Fisher, Z. Werb, *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113*, E1343.
[35] A. Gonneaud, C. Jones, N. Turgeon, D. Levesque, C. Asselin, F. Boudreau, F. M. Boisvert, *Sci Rep* **2016**, *6*, 38195.
[36] A. Cristobal, H. W. van den Toorn, M. van de Wetering, H. Clevers, A. J. Heck, S. Mohammed, *Cell Reports* **2017**, *18*, 263.
[37] S. F. Boj, C. I. Hwang, L. A. Baker, I. I. Chio, D. D. Engle, V. Corbo, M. Jager, M. Ponz-Sarvise, H. Tiriac, M. S. Spector, A. Gracanin, T. Oni, K. H. Yu, R. van Boxtel, M. Huch, K. D. Rivera, J. P. Wilson, M. E. Feigin, D. Ohlund, A. Handly-Santana, C. M. Ardito-Abraham, M. Ludwig, E. Elyada, B. Alagesan, G. Biffi, G. N. Yordanov, B. Delcuze, B. Creighton, K. Wright, Y. Park, F. H. Morsink, I. Q. Molenaar, I. H. Borel Rinkes, E. Cuppen, Y. Hao, Y. Jin, I. J. Nijman, C. Iacobuzio-Donahue, S. D. Leach, D. J. Pappin, M. Hammell, D. S. Klimstra, O. Basturk, R. H. Hruban, G. J. Offerhaus, R. G. Vries, H. Clevers, D. A. Tuveson, *Cell* **2015**, *160*, 324.
[38] S. E. Ong, I. Kratchmarova, M. Mann, *J Proteome Res* **2003**, *2*, 173.
[39] M. Mann, *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 952.
[40] S. E. Ong, B. Blagoev, I. Kratchmarova, D. B. Kristensen, H. Steen, A. Pandey, M. Mann, *Mol Cell Proteomics* **2002**, *1*, 376.
[41] T. C. Walther, M. Mann, *J Cell Biol* **2010**, *190*, 491.
[42] S. P. Gygi, B. Rist, S. A. Gerber, F. Turecek, M. H. Gelb, R. Aebersold, *Nat. Biotechnol.* **1999**, *17*, 994.
[43] P. J. Boersema, R. Raijmakers, S. Lemeer, S. Mohammed, A. J. Heck, *Nat. Protoc.* **2009**, *4*, 484.
[44] V. Vidova, Z. Spacil, *Anal Chim Acta* **2017**, *964*, 7.
[45] S. A. Gerber, J. Rush, O. Stemman, M. W. Kirschner, S. P. Gygi, *Proc Natl Acad Sci USA* **2003**, *100*, 6940.
[46] N. M. Griffin, J. Yu, F. Long, P. Oh, S. Shore, Y. Li, J. A. Koziol, J. E. Schnitzer, S. R. Finkbeiner, D. R. Hill, C. H. Altheim, P. H. Dedhia, M. J. Taylor, Y. H. Tsai, A. M. Chin, M. M. Mahe, C. L. Watson, J. J. Freeman, R. Nattiv, M. Thomson, O. D. Klein, N. F. Shroyer, M. A. Helmrath, D. H. Teitelbaum, P. J. Dempsey, J. R. Spence, *Nat. Biotechnol.* **2010**, *28*, 83.
[47] W. J. Qian, J. M. Jacobs, T. Liu, D. G. Camp, 2nd, R. D. Smith, *Mol Cell Proteomics* **2006**, *5*, 1727.
[48] V. Dakic, J.M. Nascimento, R.C. Sartore, R. de Moraes Maciel, D. B. de Araujo, S. Ribeiro, D. Martins-de-Souza, S.K. Rehen, *bioRxiv* **2017**, 108159.
[49] S. Haider, R. Pal, *Curr Genomics* **2013**, *14*, 91.
[50] S. R. Finkbeiner, D. R. Hill, C. H. Altheim, P. H. Dedhia, M. J. Taylor, Y. H. Tsai, A. M. Chin, M. M. Mahe, C. L. Watson, J. J. Freeman, R. Nattiv, M. Thomson, O. D. Klein, N. F. Shroyer, M. A. Helmrath, D. H. Teitelbaum, P. J. Dempsey, J. R. Spence, *Stem Cell Reports* **2015**, *4*, 1140.
[51] D. Grun, A. Lyubimova, L. Kester, K. Wiebrands, O. Basak, N. Sasaki, H. Clevers, A. van Oudenaarden, *Nature* **2015**, *525*, 251.
[52] H. Miyoshi, K. L. VanDussen, N. P. Malvin, S. H. Ryu, Y. Wang, N. M. Sonnek, C. W. Lai, T. S. Stappenbeck, *EMBO J.* **2017**, *36*, 5.

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim