Label-free mass spectrometry exploits dozens of detected peptides to quantify lamins in wildtype and knockdown cells

Joe Swift, Takamasa Harada, Amnon Buxboim, Jae-Won Shin, Hsin-Yao Tang, David W. Speicher, and Dennis E. Discher

Molecular Cell Biophysics Laboratory, University of Pennsylvania, Philadelphia PA USA, ‘Center for Systems and Computational Biology, Wistar Institute, Philadelphia PA USA

Label-free quantitation and characterization of proteins by mass spectrometry (MS) is now feasible, especially for moderately expressed structural proteins such as lamins that typically yield dozens of tryptic peptides from tissue cells. Using standard cell culture samples, we describe general algorithms for quantitative analysis of peptides identified in liquid chromatography tandem mass spectrometry (LC-MS/MS). The algorithms were foundational to the discovery that the absolute stoichiometry of A-type to B-type lamins scales with tissue stiffness (Swift et al., Science 2013). Isoform dominance helps make sense of why mutations and changes with age of mechanosensitive lamin-A,C only affect “stiff” tissues such as heart, muscle, bone, or even fat, but not brain. A Peak Ratio Fingerprinting (PRF) algorithm is elaborated here through its application to lamin-A,C knockdown. After demonstrating the large dynamic range of PRF using calibrated mixtures of human and mouse lysates, we validate measurements of partial knockdown with standard cell biology analyses using quantitative immunofluorescence and immunoblotting. Optimal sets of MS-detected peptides as determined by PRF demonstrate that the strongest peptide signals are not necessarily the most reliable for quantitation. After lamin-A,C knockdown, PRF computes an invariant set of “housekeeping” proteins as part of a broader proteomic analysis that also shows the proteome of mesenchymal stem cells (MSCs) is more broadly perturbed than that of a human epithelial cancer line (A549), with particular variation in nuclear and cytoskeletal proteins. These methods offer exciting prospects for basic and clinical studies of lamin-A,C as well as other MS-detectable proteins.

Introduction: Mass Spectrometry Uncovers Scaling of Lamina Composition versus Tissue Stiffness

The discovery that cells in culture recognize and respond to the flexibility of their substrates prompted a hypothesis in the field that such mechanosensing by cells might somehow relate to tissue elasticity. Brain tissue is normally very soft; muscle is stiffer, and bone is rigid; moreover, some stem cells respond in vitro to such differences with expression of characteristic lineage markers. In efforts to identify a general mechanism for such mechanosensing, we conducted proteomic analyses of human and mouse adult tissues, primary cells, and cell lines. For quantitation, we developed general algorithms for label-free analyses of peptides identified in now-standard liquid chromatography tandem mass spectrometry (LC-MS/MS). Among the hundreds of different intracellular proteins that we could quantify, the nuclear protein lamin-A,C was discovered as one of the very few proteins that increased systematically with tissue stiffness. Whereas the B-type lamins varied little between tissues, relative levels of lamin-A,C were lowest in a soft tissue such as brain and were up to 30-fold higher in stiff tissues such as striated muscle and bone. The goal

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Abbreviations: LC, liquid chromatography; MS, mass spectrometry; PRF, peptide ratio fingerprinting; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; KD, knockdown; A549, human epithelial cancer cell line; C2C12, mouse myoblastic cell line; MSC, mesenchymal stem cell

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*Correspondence to Dennis E. Discher; Email: discher@seas.upenn.edu

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Localization of lamin A to the eukaryotic nuclear envelope was first achieved with antibodies in the late 1970s,13 with a cell-type dependent expression of lamin A reported shortly thereafter.14 While lamin-B is expressed at relatively similar levels across solid tissues, including brain, lamin-A,C is low in the brain,15 and we would postulate that this is because lamin-A,C is the cell lines, using label-free mass spectrometry (MS) methods to provide absolute stoichiometry for the A- and B-type lamins.4 MS methods can also exploit other proteins and avoid possible limitations of antibodies such as epitope masking.16 The role of lamin-A in maintaining the structural integrity of the nucleus had long been postulated,23 and through past measurements of tissue stiffness properties we link the composition of the nuclear lamina and the mechanical properties of the nucleus to the stiffness—and therefore stress—present in the source tissue. Our MS investigations also uncovered a possible mechanism in terms of phospho-regulation of lamin-A,C levels.24 While it has long been known that lamin-A,C can be reversibly phosphorylated as the lamina is dissolved during mitosis,24 phosphorylated laminae are also present during interphase.25 MS results indicate that, when the lamina is relaxed, lamin-A,C is increasingly phosphorylated on at least four different sites and thereby solubilized and degraded, as would be the case in soft brain or circulating blood. The discovered mechanism of mecano-sensitive, protein-level regulation was further found to feedback into LMNA transcription through a retinoic acid receptor pathway of well-known importance in development and differentiation. Quantitative MS was thus a critical tool in clarifying the mecano-sensitive basis for variations in lamin-A,C.

MS can independently detect many peptides from the same protein, and in the case of lamin-A,C the sequence is often covered by antibodies such as epitope masking in identification and the potential to analyze altered post-translational states in tissues and perhaps mutations. Although a large number of lamin specific antibodies have been produced, spanning the entire length of the protein and including regions conserved across all lamins,27 such a library of affinity probes is not always widely available nor does it seem practical to employ such a library in large studies. Antibodies do not yet exist for many post-translational states, including phosphorylations, acetylations, and in situ synthetic modifications of cysteines that we used to probe the conformation of the immunoglobulin-like domain in lamin-A,C.28 MS methods can also exploit peptides shared by isoforms to quantify absolute stoichiometry of protein isoforms. We have therefore developed MS approaches to characterize and quantify the lamins in all of these capacities.

Here we describe in detail an application of label-free MS analysis to cells subjected to siRNA-mediated knockdown of lamin-A,C. We take advantage of the many peptides typically detected for such a relatively abundant protein, optimize for proportionality in protein quantitation within and between samples, and generate a wealth of additional information including the identification of a “best set” of housekeeping proteins and broader perturbations to the proteome. Knockdown approaches now pervade cell biology and are emerging in therapeutics29, but biological variations in lamin-A,C have been reported as cancer biomarkers in patients,30 during aging31 and stem cell differentiation.32 Here, lamin-A,C was partially knocked-down in a human lung cancer derived cell line (A549) that report endogenous some characteristics of con- fmer stem cells, and it was knocked down in human MSCs. Our studies combine label-free mass spectrometry methods with antibodies in the late 1970s,13 with a cell-type dependent expression of lamins reported shortly thereafter.14 While lamin-B is expressed at relatively similar levels across solid tissues, including brain, lamin-A,C is low in the brain,15 and we would postulate that this is because lamin-A,C is the cell lines, using label-free mass spectrometry (MS) methods to provide absolute stoichiometry for the A- and B-type lamins.4 MS methods can also exploit other proteins and avoid possible limitations of antibodies such as epitope masking.16 The role of lamin-A in maintaining the structural integrity of the nucleus had long been postulated,23 and through past measurements of tissue stiffness properties we link the composition of the nuclear lamina and the mechanical properties of the nucleus to the stiffness—and therefore stress—present in the source tissue. Our MS investigations also uncovered a possible mechanism in terms of phospho-regulation of lamin-A,C levels.24 While it has long been known that lamin-A,C can be reversibly phosphorylated as the lamina is dissolved during mitosis,24 phosphorylated laminae are also present during interphase.25 MS results indicate that, when the lamina is relaxed, lamin-A,C is increasingly phosphorylated on at least four different sites and thereby solubilized and degraded, as would be the case in soft brain or circulating blood. The discovered mechanism of mecano-sensitive, protein-level regulation was further found to feedback into LMNA transcription through a retinoic acid receptor pathway of well-known importance in development and differentiation. Quantitative MS was thus a critical tool in clarifying the mecano-sensitive basis for variations in lamin-A,C.
with an algorithm developed to identify optimal multi-peptide fingerprints by filtering out the distorting effects of incomplete trypsinization, peptides shared between isoforms, variable ionization, and overlapping signals. Although not as accurate and reproducible as isotope labeling schemes, label-free methods have already been described by others—with recent improvements in separation technology and MS instrumentation—to offer a compelling alternative to immunoblotting for protein quantification on bulk lysates. The ratiometric scheme described herein could also be applied to isotopically labeled data sets—whether labeling is done by metabolic incorporation of amino acids containing heavy isotopes or by chemical modification with mass-tagged labels. In general application to studies of any bulk lysates, we routinely begin with this ratiometric algorithm and then try to find commercial antibodies that yield similar insight by immunoblot and/or quantitative immunofluorescence. The following illustrates the consistency that can be achieved—and for an extremely interesting protein.

**Ratio-of-Ratios Optimization for Proportional Signals**

For analysis of complex protein samples, LC-MS/MS has emerged as a means to both discover and catalog peptides derived from a well-defined and predictable tryptic digestion of proteins. In the experiments detailed here, a single sample injection followed by a ~80 min LC elution and MS/MS analysis leads to identification of several thousand tryptic peptides, typically allowing hundreds of proteins to be identified (depending on the molecular weight and charge state of the peptides).
weight range). As there is no “amplification” process in proteomic analysis, methods are dependent on maximizing separation and signal while suppressing background. Thus greater fractionation of a sample, for example by analyzing smaller ranges of molecular weight (to detect low copy number proteins) or using orthogonal separation schemes (for example, to enrich for membrane proteins), leads to a greater depth of analysis. The most advanced methods can approach near-complete coverage of the proteome, but such depth is not necessary to quantify the relatively abundant structural proteins described here.

Each trypic peptide is quantified by its ion current in the first MS and all trypic fragments from the same parent protein should be present at the same concentration and should maintain proportionality between samples, but ion currents differ for many reasons—including spliceform variations and inefficiencies in trypsinization, separation, or ionization. While labeling methods designed for protein quantification are now common in mass spectrometry, the most robust for protein quantitation remain an important issue.

Our “Peptide Ration Fingerprint” (PRF) algorithm identifies the best peptides—from a target protein (lamin-A,C here) or from any candidate standardizing protein—that maintain proportionality between peptides for different data sets. All possible peptide-pair ion current ratios obtained from knockdown vs. wildtype conditions are plotted against each other for lamin-A,C in a log-log scatterplot (Fig. 1B), and the
deviation from ideal, proportional behavior is revealed by the distance from the diagonal, with the optimized set selected such that $R^2 > 0.998$. Extensive statistical studies have assessed the confidence in protein assignments made by MS, but in identifying the best peptide set with which to compare samples, the PRF approach is not blindly statistical as it provides quality control by selection based on a physical expectation of peptide behavior, even when signal magnitude varies greatly between peptides. Differences in total concentration between samples are likewise unimportant. Constitutive “housekeeping genes” used for normalization in quantifying mRNA by real-time polymerase chain reaction typically include: GAPDH, albumin, β- and γ- actins, α- and β- tubulins, cyclophilin, hypoxantine phosphoribosyltransferase and L32. A “housekeeping protein” might also be chosen for normalization of both MS and Westerns, but care must be taken when assuming a protein will remain constant. For example, GAPDH levels vary significantly when estrogen levels vary and when cells approach confluency. LC-MS/MS allows far more flexibility when choosing suitable normalization, and with the PRF optimization scheme, we normalize against a peptide set that is seen to be invariant between conditions (Fig. 1c) by randomly sampling the entire peptide population (or assessing all peptide-pairs if computational resources are not limiting). These optimized peptide subsets are derivative of a set of housekeeping proteins which, in use here, must be trypsinized consistently, ionized reproducibly, etc. The optimal subset nonetheless spans a dynamic signal range similar to the full set of peptides. Furthermore, peptide ratios that deviate significantly from the diagonal illustrate the non-Gaussian nature of systematic errors intrinsic to some aspects of the MS process (e.g., peak overlap or misidentification).

To determine the extent of knockdown of a target, all target-to-control peptide ratios are plotted for knockdown vs. wildtype (Fig. 1D) with the intercept revealing the extent of knockdown. Partial knockdowns, such as of lamin-A,C here, are typical of siRNA studies and thus present an ideal challenge to test quantification by MS compared with established methods (such as western blot, Figure 1D, inset). In this example (lamin KD with siRNA in A549 cells), quantification by MS was found to agree well with western blotting, although it should be noted here that discrepancies can arise in immunoblotting due to the inherent nonlinearity of response to concentration (Fig. S2). Optimal Peptides for Quantitation are Not Necessarily Detected with High Signal

Knockdown was calculated from large random samples of lamin peptides, and comparing the results for small sets both with and without optimization of the lamin peptide set illustrates the importance of increased sampling (Fig. 1E).
Larger sample sizes produce an exponential reduction in uncertainty, and with optimization through elimination of anomalous peptides the increase in the number of peptides required to halve the standard deviation was reduced by about 25%. The knockdown also converged to a 3% higher value as anomalous peptides were removed from the test set.

Choosing peptides by hand is subjective and time consuming, especially when extended to a multi-sample, global proteome-scale study. Tryptic peptides might originate from more than one protein or isoform, and some peptides are intrinsically less suited to consistent detection in LC-MS/MS for reasons of size, solubility, LC elution, or ionization propensity. Inspection of elution profiles for artifacts (e.g., overlapping peaks) is also tedious. Surprisingly, selection of peptides based on strong signals fails as well: Figure 1F shows that the ion current distribution for the optimal peptides is similar in form and shifted minimally to higher ion current when compared with the distribution for all peptides in a data set. As such, PRF optimization does not preferentially select for high signal strength.

Although it is not necessary to analyze the properties of individual peptides, we find (Fig. S3) that discarded peptides might be: (1) common to more than one protein, (2) subject to inconsistent trypsin cleavage, or (3) have poor LC peak alignment or peak overlap. Peptides could also be removed if they have been misidentified or if the extent of a post-translational modification varies between experimental conditions. Note that it is only a change in modification that would be problematic: a peptide that is always modified, such as one containing a cysteine alkylated as part of the sample preparation, could be suitable for quantification. Conversely, a peptide that is extensively modified, for example by phosphorylation, in response to the perturbation between samples (i.e., KD vs. WT) would be unsuitable, although in the case of lamin-A,C here, we find the extent of phosphorylation to be limited. This filtering method is effectively orthogonal to other established quality control methodologies, such as selection by signal magnitude, mass error or deltaCn.

Mixed Species and/or Tissue Samples Demonstrate Large Dynamic Range of MS Quantification

To assess the accuracy of PRF within a complex sample—of particular relevance to tissue analyses of laminins, as well as to co-cultures of human stem cells with mouse mesenchyme-derived “feeder” layers and any xenobiotic sample (e.g., fetal calf serum in human cell cultures)—we analyzed a mixture of human (A549) and mouse mesenchyme-derived myoblastic cells (C2C12 progenitor line) at ratios between 10% and 90%. Detected tryptic fragments gave ~60% common peptides plus peptides unique to each species that were used for species-specific quantification. As proof of the label-free, PRF-optimized quantification, we measured the change in each protein level between mixed-lysate samples relative to a normalizing peptide set drawn from the opposite species. So, for example, in comparing proteins from a 25%-mouse, 75%-human sample to a 50%-mouse, 50%-human sample we expect, based on the titration, to measure a change to (25/75)/(50/50) = 33%. This process was repeated for all sample ratios in each of three molecular weight bands, considering all mouse and human proteins detected with more than three species-unique peptides and/or protein, effectively giving 572 discrete checks of the method’s veracity (Fig. 2A lists structural proteins as an example). In addition, we considered a repeat analysis of the same sample, in this case expecting all proteins to be maintained at 100% (we used a duplicate analysis of an A549 lysate, which is not shown). This method is orthogonal to other quality control methodologies, such as selection by signal magnitude, mass error or deltaCn.
allowing quantification of 244 proteins; Fig. 2B shows structural proteins).

Figure 2C shows the distribution of proteins quantified in this experiment: agreement with titration is generally good across the measured dynamic range of almost 100-fold (between 11% and 900% of the normalization sample), with the greatest deviation observed at the extremes. Based on sample sizes and dilution analyses, we estimate a lower sensitivity limit of ~10^4 cells for lamin-A,C quantitation by MS. Modern LC-MS/MS instruments can detect proteins down to low femtomole to high attomole levels, and although sequence coverage becomes limiting, this sensitivity makes MS well-suited to studies of primary, patient-derived stem cells that are often slow-growing and limited in supply.

**Comparison to Immunoblotting, Immunofluorescence, and Transcript**

We typically find good agreement between protein quantification by immunoblotting (Westerns) and MS methods (e.g., Fig. 2D), although care must be taken in blotting for suitable normalization schemes and when using a linear approximation to the relationship between protein concentration and band density. Of course, MS and immunoblotting both rely on an effective solubilization of protein prior to SDS-PAGE. Control experiments were therefore run to ensure that all lamin was successfully separated in the gel with our solubilization protocol (Fig. S4). In addition to these methods, protein concentration is commonly determined by immunostaining and microscopy. We compared measurements of lamin-A KD in a culture of MSCs by MS, immunoblotting, immunofluorescence and transcriptional profiling (Fig. 3). All methods agreed on a KD to ~35% relative to untreated cells, with the exception of immunofluorescence which underestimated the extent of knockdown. We hypothesize that this difference is because the antibody has easier access to the lamin in the depleted knockdown lamina, meaning that a greater fraction of lamin proteins are fluorescently tagged in this sample. This is perhaps an example of “epitope masking,” which can complicate accurate quantification by antibody methods.

**Proteome Profiling Following Lamin KD in A549 and MSCs**

MS allows a large number of proteins to be examined in the same experiment in lieu of working with dozens of antibodies. Lamin was knocked down by siRNA (two alternative siRNAs were used either individually or in combination, Fig. S5) in A549 and MSC cells and lysates analyzed by MS. The PRF algorithm was used to measure the extent of perturbation to the proteome concurrent with lamin knockdown by quantifying changes in all proteins detected with three-or-more peptides and/or protein. The PRF algorithm was used to experimentally determine peptide sets for normalization in each of the molecular weight bands (i.e., representative of invariant “housekeeping” proteins, Fig S6). As normalization proteins in MS, there is an added implication of reliability in trypticization, elution from PAGE gels, robust MS signals, etc. A number of heat shock proteins were determined to be appropriate standards within the lamin band for the perturbations applied here (HSPA5, HSPA8, HSP90B1), with the constitutively expressed heat shock protein HSP-90B high in the list, consistent with recent reports. The proteins that make the biggest contribution to the peptides in the invariant set (HSPs, actins, calnexin, etc.) show the least variation between samples—consistent with housekeeping proteins.

Histograms of the changes in the detectable proteome (455 proteins) are shown for both cell lines in Figure 4 (all data are shown in Fig. S7). In agreement with recent findings, the perturbation to the A549 proteome is relatively slight in response to a lamin KD to ~30% of WT. The perturbation is broader in MSCs in response to a KD of similar magnitude. A breakdown of detectable proteins by function indicates that perturbation to the proteome in MSCs is primarily focused in two areas: upregulation of nuclear proteins (typically proteins involved with mRNA splicing and processing, chromatin binding proteins, and helicases) and down-regulation of cytoskeletal and structural proteins (typically actin binding proteins). That these groups are most perturbed is perhaps unsurprising: the nucleus is known to be an important mechanical component of the cell, with the lamins interfacing with other structural components through the LINC (linker of nucleoskeleton and cytoskeleton) complex. It has been reported that lamin-A,C level can influence actin-bundling proteins such as T-plastin. Many cytoskeletal proteins are regulated by the SRF pathway, which itself is influenced by actin, known to interact directly with lamin-A,C and other nuclear proteins such as emerin. In addition, there is increasing evidence that lamin interacts with chromatin, transcription factors and influence

![Figure 5: Schematic of method used to determine the absolute stoichiometric ratio (φ) between proteins](image)
nuclear signal transduction pathways. Therefore, the PRF method allows more complete, yet quantitative analysis of what proteins are perturbed upon knockdown, making it useful for the systems biology field to construct regulatory circuits from gene to protein.

**Determination of Absolute Isoform Ratios**

The high sequence coverage of lamins by MS (Fig. 5B) makes it possible to identify peptides common to both LMNA spliceoforms (i.e., lamin-A and lamin-C), peptides unique to lamin-A or lamin-C, peptides unique to lamins B1 and B2, plus a peptide common to all lamins. The ability to detect both unique and overlapping regions of the protein sequence between isoforms or spliceoforms allows derivation of the absolute ratio between the proteins (general scheme shown in Fig. 5). These equations can be used in a number of ways to study lamins: in determining the ratio between lamins A and C, we find general agreement with immunoblotting (data not shown). However, this measurement is highly dependent on effective detection of unique lamin A and C regions, that yield only three and one peptides respectively. In this case, therefore, densitometry is the superior method of measurement, assuming that the antibody against lamin-A,C binds both spliceoforms equally.

Intriguingly, we often find that lamin A is knocked down to a greater extent than the common lamin-A,C region. Although we don’t always find that the single lamin C specific peptide is correspondingly less perturbed, we consider this measurement less reliable as it comes from one peptide. As the siRNA targets both spliceoforms of lamin in the same way, we can interpret this observation in two ways: (1) lamin-A could be turned-over faster in the nucleus so the lack of newly transcribed, replace-ment protein is more pronounced, and/or (2) following KD, a feedback mechanism within the cell drives the lamin-C to be replaced at a greater rate. Time-resolved KD experiments should help elucidate such mechanisms.

We found the absolute ratio between A-type and B-type lamins to be characteristic of nuclear mechanical properties and to scale with tissue stiffness. In making these measurements by MS, the sequence regions unique to lamin-A,C and lamins B1 and B2 can readily be quantified (typically detected with dozens of peptides), but the overlap region is a single tryptic peptide, LLEGEEER. We have previously established detection and linear MS response of LLEGEEER through the titration of a synthetic peptide. However, once the absolute A:B ratio has been established in a standard sample, further measurements of A:B can be made relative to this without further need for detection of the overlap peptide. Here the absolute A:B ratio was determined with high confidence in A549 cells from lamin-A knockdown experiments (see calculations in Table S1): lamin-A:B = 2.5 ± 0.4 (n = 3). The lamin-A:B ratio in the corresponding lamin KD in A549 was 3.0 ± 0.2. Absolute lamin-A:B ratios in other cell lines follow from these standardizing numbers, often where low sample abundance, for example in primary cell lines, would make direct detection of LLEGEEER difficult: mouse embryonic stem cells, 0.10 ± 0.02 (note that although this is low, we cannot rule out contamination from other cell populations: embryonic stem cells are thought to contain minimal lamin-A); circulating myeloid blood cells, 0.6 ± 0.1 (from data in Shin et al.); human brain glioblastoma line U251, 0.8 ± 0.2; C2C12 cells, 4.6 ± 0.8; MSCs, 10 ± 2.

**Conclusion:** Mass Spectrometry is a Powerful Complement to Antibody Methods

The type of analyses here of lamins by label-free MS methods led to our discovery that the absolute stoichiometry of A-type to B-type lamins scales with tissue stiffness. This finding was followed up with immunoblots of tissues and then various antibody and GFP fusion studies of mechanism sensitive lamin-A in cell culture models. Our label-free MS analyses were far from exhaustive, with lamin-A,C emerging from quantitation of just hundreds of proteins in tissue, and so there might be other intracellular proteins that exhibit equally interesting trends with tissue stiffness (in addition to extracellu-lar collagens that we also described ). Regardless, given the many basic and clinical studies of lamin-A and many other MS-detectable proteins, these new and powerful methods are a valuable addition to the protein analysis toolkit. They can also be used in conjunction with variations of other antibody-based methods, such as flow cytometry.

**Disclosure of Potential Conflicts of Interest**

No potential conflict of interest was disclosed.

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**Supplemental Materials**

Supplemental materials can be found here: www.landesbioscience.com/nucleus/article/27413
35. Korsbie G, Dahmus MG, Franz W. W20 Cell-type specific differences in protein composition of nuclear pore complex-lamina structures in vertebrate nuclei. J Cell Biol 1997, 139: 207-217; PMID:9204934; http://dx.doi.org/10.1083/jcb.139.1.207

36. Wills ND, Cox TR, Rahman-Canal S, Steven K, Praetorius MA, van den Brand P, van Engeland M, Weijenberg M, Wilson DE, de Boer A, Elmen J. Lamin A/C is a risk biomarker in colorectal cancer. PLoS One 2009; 4:e3517; PMID:19358556; http://dx.doi.org/10.1371/journal.pone.0003517

37. Ten Blom H, Kikkeri S, Satyanarayana R, Smit M, van der Gaag GC, van der Plas J, Willemse S, de Looij E, van Rijn SC, Dirksen D. Tissue stiffness and mechanical anisotropy in normal and neoplastic human tissues. J Exp Clin Cancer Res 2011; 30: 226; PMID:21755506; http://dx.doi.org/10.1186/1756-9966-30-226

38. Selb-Perelman Y, Silberstein M, Cohen O, Auluck PK, Leder P. Lamin A/C is required for proliferation and viability of colorectal cancer cells. Proc Natl Acad Sci U S A 2011; 108:18883-18888; PMID:22014032; http://dx.doi.org/10.1073/pnas.1108564108

39. Kim Y, Suárez AM, Abbott KE, Shao H, Inan A, Warkentin MA, Su H, Gao Z, Mebius RE. Brachyury is required for proper angiogenesis but not by endothelial cells. Sci. 2011; 331:570-574; PMID:21490694; http://dx.doi.org/10.1126/science.1200569

40. Sánchez-Gómez J, Alba M, Elías M. Immunocytochemical localization of lamins in murine mammary gland. J Cell Biol 1982, 95:755-764; PMID:717680; http://dx.doi.org/10.1083/jcb.95.2.755

41. Shiga T, Okuda H, Sugi T, Minoura S, Banno K, Ueda Y, Okada S. Analysis of laminopathies in human aging. Science 2006; 312:1059-1064; PMID:16884226; http://dx.doi.org/10.1126/science.1126892

42. Ong SE, Mann M. A general approach for in vivo and in vitro labelling by amino acids in cell culture (SILAC). Nat. Methods 2004, 1:95-99; PMID:15216666; http://dx.doi.org/10.1038/nmeth1004-95

43. Pelham RJ Jr., Wang YL. Cell locomotion and integrin adhesions are regulated by substrate flexibility. J. Cell Biol. 2008; 181:627-638; PMID:18511624; http://dx.doi.org/10.1083/jcb.200802087

44. Lewensohn R, Lehtiö J. Robustness and accuracy of high speed LC-MS separations for global peptide quantitation and biomarker discovery. J Chromatogr B Analyt Technol Biomed Life Sci 2010; 897:1-13; PMID:20728833; http://dx.doi.org/10.1016/j.jchromb.2009.02.052

45. Galvez BV, Chandley AC, Nzwawe M, Bijlsma GW, Cockshott E. Expression of the nuclear lamina in normal and neoplastic human tissues. J Exp Clin Cancer Res 1992; 11:235-246.
42. Groe S, Honeywell R, Gallo M, Andaluzad B, Raptop L. Housekeeping genes: expression levels may change with density of cultured cells. J Immunol Methods 2010; 355:76-9; PMID:20771669; http://dx.doi.org/10.1016/j.jim.2010.04.036

43. Peters A, Pan Y, Proust S, Skalli O. On the use of ratio standard curves to accurately quantify relative changes in protein levels by Western blot. Anal Biochem 2010; 397:395-9; PMID:20808999; http://dx.doi.org/10.1016/j.ab.2010.04.019

44. Chavara N, Lambeth M, Nakano P, Landry J. Protein quantification by bioluminescence Western blotting: elimination of the antibody factor by dilution series and calibration curve. J Immunol Methods 2010; 355:140-6; PMID:20897799; http://dx.doi.org/10.1016/j.jim.2010.04.036

45. Afflak J, Tabb D, Chaffarier I, Ryan D, Attie R, Laroo R, Dugan K, Dugan G. Age-related change in lamin A/C expression in cardiomyocytes. Am J Physiol Heart Circ Physiol 2007; 293:H1481-6; PMID:17513488; http://dx.doi.org/10.1152/aphp.00194.2006

46. Mizuno M, Chacrin MR, Yoda F, Maga A, Ruhart C, Vidas S, Sannoy M, Okum C, Virdun J. LMNA messenger RNA expression in highly active antiretroviral therapy-treated HIV-positive patients. J Acquir Immune Defic Syndr 2007; 46:386-9; PMID:18077642; http://dx.doi.org/10.1097/QAI.0b013e3180e5eb1f

47. Chen S, Martin C, Maya-Mendoza A, Tang CW, Levis J, Saez PE, Jackson BA. Reduced expression of lamin A/C results in modified cell signaling and metabolism coupled with changes in expression of structural proteins. J Proteome Res 2009; 8:916-21; PMID:19775189; http://dx.doi.org/10.1021/pr900354v

48. Dral KN, Rothen-Gaedd EA, Lauber R. In the middle of it all: mutual mechanical regulation between the nucleus and the cytoskeleton. J Biomech 2007; 40:1084-9; PMID:17934806; http://dx.doi.org/10.1016/j.jbiomech.2006.12.007

49. Olson EN, Neubosch A. Linking actin dynamics and gene transcription to drive cellular multidfunction. Nat Rev Mol Cell Biol 2008; 9:355-65; PMID:1844257; http://dx.doi.org/10.1038/nrm2390

50. Simon DN, Zimmerman MS, Wilson KL. Direct actin binding to A- and B-type lamin tails and actin filament binding by the lamin A tail. Nucleus 2010; 1:264-72; PMID:20327874; http://dx.doi.org/10.4161/nucle.1.3.11799

51. Hu CY, Jaddick DE, Vandersluis ME, Lammersen J. Lamin A/C and emerin regulate MRLK-ERK activity by modulating actin dynamics. Nat Rev 2003; 497:1073-11; PMID:20644198; http://dx.doi.org/10.1038/nature03205

52. Andrits N, Gruolke JM. Role of inner lamin in signaling, transcription, and chromatin organization. J Cell Biol 2009; 187:493-50; PMID:19834675; http://dx.doi.org/10.1083/jcb.200904124

53. Shkol P, Smith J, Spiro J, Rice DE. Muscle II antibodies and soft 2D matrix transverse multilamellar and cellular projections typical of planar-producing megakaryocytes. Proc Natl Acad Sci U S A 2011; 108:10598-603; PMID:21703232; http://dx.doi.org/10.1073/pnas.1017479108