A gene's mRNA level does not usually predict its protein level

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Abstract

Since publication of the complete human genome sequence in 2004, genomic approaches have dominated biomedical research. This is despite the fact that the morphology of 200+ human cell types, all with the same genome, varies profoundly because of differing protein expression. The question arises: can protein amount be deduced by measuring mRNA concentration? Evidence is presented here from five different mass spectrometry groups that the answer is mostly no. The average R^2 or R_s correlation coefficient from 20 plots of mRNA versus protein concentration (from six kinds of mammalian cultured cells and twelve human tissues) is 0.40 +/- 0.06. That is, agreement between the two measurements occurs about 40% of the time. Further evidence of discrepancies between mRNA and protein levels in vivo is presented from six studies of human patients including 1) somatostatin receptor 5 and chemokine receptor 7 in Crohn's disease, 2) the JAG1 gene in breast cancer, 3) the p53, BCL-2 and BAX genes in breast cancer, 4) CD20 antigen in chronic lymphocytic leukemia cells, 5) matrix metalloproteinases in prostate cancer, and 6) cytokines in influenza A vaccination studies. The conclusion is clear: mRNA levels cannot be used as surrogates for corresponding protein levels without verification.

Introduction

Genomics dominates biomedical research: The 21st century began with a most magnificent success: publication of the human genome sequence of 2.85 billion base pairs. After two preliminary papers by HGC (International Human Genome Sequencing Consortium) and Celera in 2001, the finalized publication in 2004 (HGC) provided 99.7% of the sequence with only 1 error/100,000 bases. [1], www.genome.gov/12513430 This colossal feat has influenced all subsequent biomedical research [2]. A multitude of high-throughput tools have since been developed to expand and compare genomes and mRNA. Genome sequences of >250 eukaryotes and >4000 bacteria and viruses have been published; BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) is providing a giddying amount of information, especially with regard to evolution.

The problem is that human tissue is comprised of protein: Although RNA was the primordial molecule, proteins are the stuff of life now. The >200 cell types in the human body have the same DNA yet they take different shapes and functions because of varying protein composition. Unfortunately, the latter is hard to figure out. The human genome codes for ~20,500 proteins [3]; alternative splicing [4] and post-translational modifications create many additional variants of physiological importance. Since the 2004 milestone, many biomedical researchers assume that proteins, difficult to measure, are proportional to the levels of corresponding mRNA, easy to measure. *Unfortunately, this intuitive assumption is wrong. Only about 40% of cellular protein levels can be predicted from mRNA measurements.*

What is the evidence that protein levels are not proportional to mRNA? Since 2004, at least 4 groups have published data on the global correlation between protein and mRNA concentration on mammalian cultured cells using mass spectrometry to quantify proteins. Their correlation results are either expressed in terms of the coefficient of determination (R^2) or the Spearman correlation coefficient (R_s). R^2 gives the fraction of the variability in Y that can be explained by the variability in X through their

linear relationship. R_s is similar, but is recommended for use with data that are skewed or have outliers. [5]. Results from the four groups, summarized in Table 1, are in surprisingly good agreement.

Authors	Year	cell line	Protein/RNA pairs	Methods (protein/RNA)	R^2 (R_s)
Tian et. al. (6)	2004	mouse EML cells	425	ICAT MS/microarray	0.35
Vogel et. al. (7)	2010	medulloblastoma*	512	Shotgun MS/microarray	0.29 (0.46)
Lundberg et. al. (8)	2010	brain glioblastoma*	5,197	triple SILAC MS/RNA sequencing	(0.43)
Lundberg et. al. (8)	2010	squamous cell carcinoma*	5,158	triple SILAC MS/RNA sequencing	(0.42)
Lundberg et. al. (8)	2010	bone osteosarcoma*	5,210	triple SILAC MS/RNA sequencing	(0.42)
Schwanhauser et. al. (9)	2011	NIH3T3 mouse fibroblast	5,028	SILAC MS/RNA sequencing	0.37, 0.41
				Average all values +/- SD	0.39 +/- 0.05

Table 1. Summary of results for the relationship between mRNA and protein concentration in mammalian cultured cells where both measurements were performed by the authors. R^2 is the coefficient of determination while R_s is the Spearman correlation coefficient [5]. Averages taken separately for the two coefficients are R^2: 0.36 +/- 0.05 (n = 4); R_s: 0.43 +/- 0.02 (n = 4.) Mass spectrometry (MS) was used to determine protein concentration in every case, either by SILAC (Stable isotope labeling by amino acids in cell culture), ICAT (Isotope-coded affinity tag) or shotgun methods. mRNA concentrations were determined by either microarrays or RNA sequencing. * human cell lines. Results from Table 1 are described in more detail below.

Tian et. al. [6] in 2004 measured steady state levels of protein (ICAT/MS) and mRNA (Agilent microarrays) in multipotent mouse EML cells and their differentiated progeny MPRO cells. The abundance ratios of 425 proteins were mapped to the corresponding mRNA expression levels. Signature genes (150) were identified that showed significant changes at either protein and/or mRNA level between the two cell types. Of these, 29 (19%) showed good correlation between mRNA and protein levels, 67 (45%) showed significant changes at the mRNA but not the protein level, and 52 genes (35%) showed significant changes at the protein but not the mRNA level. Two genes (1%) showed opposite expression patterns of mRNA and protein. The correlation coefficient R between mRNA and protein was 0.64 (R^2 = 0.41) for the signature genes and 0.59 (R^2 = 0.35) for all genes examined. Interestingly, the c-kit receptor kinase protein and mRNA expression varied 7-fold and 9-fold respectively between the two cell lines. However the c-kit *ligand* protein, aka stem cell factor, had a 5-fold higher level in the EML cells but no change in mRNA levels. Nine mitochondrial proteins showed significantly lower protein levels in the MPRO cells but higher or similar levels of mRNAs compared to those in the EML cells. Five out of six mRNA processing genes showed a negative correlation between mRNA and protein levels.

Vogel et. al. [7] in 2010 pointed out that transcription, mRNA decay, translation, and protein degradation are key processes determining steady state protein concentrations. They measured protein using shotgun MS and mRNA levels using microarrays for 1025 genes from human medulloblastoma cells harvested at steady state logarithmic growth. A high-confidence dataset of 512 genes was chosen for close examination. A plot of protein versus mRNA level for these proteins gave an R^2 value of 0.29; R_s value was 0.46. Furthermore, about 200 sequence features (coding sequence lengths, nucleotide frequencies and properties etc) were checked for correlation with protein abundance. The authors concluded that the processes of translation and protein degradation are at least as important as mRNA transcription and stability to steady-state protein abundance.

Lundberg et. al. [8] in 2010 performed a global analysis of mRNA and relative protein abundance in 3 human cancer cell lines: a brain glioblastoma (U-251MG) ; epidermoid squamous cell carcinoma (A-431); and bone osteosarcoma (U-2 OS). To measure mRNA, they used digital RNA seq, a method more specific and sensitive than microarrays,. A total of 15,538 transcripts were detected in the three cell lines of

which 11,575 (74.5%) were common to all three. Only 559, 572, and 922 transcripts were unique to the brain, epidermoid and bone cell lines, respectively.

To measure protein levels, they used a triple-SILAC (stable isotope labeling by amino acids in cell culture) method in which the three cell lines were grown with amino acids with different isotopes and then analyzed by mass spectrometry. In this method, triple peak patterns are generated for each protein; identification of one of the peptides automatically yields the identity of the other two. A total of 5456 proteins were quantified. Of these, 5333 (97.7%) were found in all three cell lines. About 65% of the detected proteins have similar expression levels (less than 2-fold differences) in the three cell lines whereas about one third had differential expression. Only 3, 27, and 34 proteins were found to be unique to the brain, epidermoid, and bone cell lines, respectively.

Although this paper focused on the differences between the cell lines, Figure S8 in supplemental data showed the 3 correlation plots between mRNA transcript and protein levels. The Spearman correlation coefficients were 0.42, 0.42 and 0.43, respectively, for bone osteosarcoma (n = 5210), epidermoid squamous cell carcinoma (n = 5158), and brain glioblastoma cells (n = 5,197).

Going further, **Schwanhausser, et. al. [9, 10] in 2011** performed a definitive global analysis of protein versus mRNA levels in NIH3T3 mouse fibroblasts. This German group used metabolic pulse labeling (SILAC) followed by mass spectrometry to measure protein turnover, and 4-thiouridine to measure mRNA turnover in exponentially growing, non-synchronized cells. They identified 6445 unique proteins of which 5279 were quantified by at least three peptide ratios. In parallel, newly synthesized RNA was pulse labeled for 2 h with 4-thiouridine. RNA samples were fractionated, analyzed by mRNA sequencing, and quantified. Both protein and mRNA half-lives were calculated. Proteins were, on average 5 times more stable (median half-life 46 h) than mRNA (9 h). There was no correlation between mRNA and protein half-lives (R^2 = 0.02.)

These authors calculated absolute cellular mRNA copy number based on number of sequencing reads in the unfractionated sample. They calculated absolute protein copy numbers from the mass spectrometry data, by summing peak intensities of all peptides matching to a specific protein. To avoid bias, they restricted their analysis to 5028 genes that were identified at both the mRNA and protein levels. In this subset, proteins were on average, 900 times more abundant than corresponding mRNAs and their concentrations spanned 5 orders of magnitude. Despite the huge spread, log-log plot of mRNA versus protein showed a R^2 value of 0.41. Removal of less reproducible data points did not improve the correlation, but rather brought R^2 to about 0.3 (Supplemental Figure S7).

Cultured cells have limits: While the studies above are in agreement about the general relationship between mRNA and protein, they were limited to cultured cells. But cultured cancer cells are almost certainly not representative of differentiated cells in the human body. The genome of Hela cells, for example, is quite abnormal, showing a high level of aneuploidy and chromothripsis [11]. *In vivo,* cells are under the influence of constant paracrine and exocrine signaling required for tissue differentiation and maintenance [12].

Wilheim et. al. recently published a remarkable draft map of the human proteome based on mass spectrometry analysis [13]. Within this paper (Extended Data Figure 7a) the authors compared another lab's newly published mRNA values determined by RNA-seq [14], with their own quantitative protein measurements for 12 human tissues. Spearman coefficients for the twelve plots of mRNA versus protein expression are shown in Table 2. The average R_s value is 0.41 +/- 0.07, not significantly different than the 0.39 +/- 0.06 average shown in Table 1 for mammalian cultured cell lines. Given the heterogeneity of the twelve tissues and the fact that mRNA measurements were determined in different individuals by different laboratories, the agreement with the cultured cell average value is remarkable. The average

value for the 20 measurements from Tables 1 and 2 combined is 0.40 +/- 0.06. If only Rs values from both tables are used the average is 0.42 +/- 0.06 (n = 16.)

tissue	R_s	tissue	R_s	tissue	R_s	Average R_s
uterus	0.34	stomach	0.45	adrenal gland	0.47	0.41 +/- 0.07
kidney	0.56	prostate	0.41	salivary gland	0.46	
testis	0.33	ovary	0.39	spleen	0.39	
pancreas	0.38	thyroid	0.31	esophagus	0.45	

Table 2. Spearman coefficients for mRNA versus protein plots for proteins in twelve tissues analyzed by Wilheim et. al. in their 2014 Nature publication [13]. The number of protein/mRNA pairs was not listed for the individual tissues. Note that these authors did not measure mRNA levels in the same tissues used for protein determinations, but rather used mRNA values from a current database.

Wilheim et. al. was focused on defining the normal human proteome. However, **measurement of protein concentration changes would be most useful for elucidation of human disease processes.** Six cases where the correlation between mRNA and protein levels was measured in cases of human diseases are described below.

1. **Taquet, et. al. [15] studied mRNA and protein expression of somatostatin receptor 5** (SSR5) and chemokine receptor 7 (CCR7) in ten Crohn's disease patients and healthy controls using PCR and IHC. Peripheral blood mononuclear cells (PBMCs) were separated using density gradient centrifugation; mucosal biopsies were obtained during colonoscopy. A significant increase in mRNA expression, 417 +/- 71 times (P <0.05), was observed for SSR5 in Crohn's disease versus controls in PBMCs. However, no increase in protein expression was detected. On the other hand, CCR7 mRNA and protein expression in mucosal biopsies were both 10-fold increased in Crohn's disease over controls. mRNA data alone would have been misleading for SSR5.

2. **Dickson et. al. [16] used in situ hybridization with an anti-sense ^{33}P-labeled cRNA probe to quantify mRNA and immunohistochemistry (IHC) to determine protein expression of the JAG1 gene in breast cancer.** The JAG1 protein activates the Notch signaling cascade that plays a role in cell differentiation and division. This group had previously showed that high levels of JAG1 or NOTCH1 mRNA were correlated with poor prognosis of breast cancer [17]. While the correlation between high JAG1 and poor prognosis held, this group found only 65% agreement between mRNA and protein levels. Patients with tumors expressing high JAG1 protein, high mRNA or both, had a 10-year survival of 31%, 19% and 11% respectively. The IHC protein measurement alone was not as good a predictor as mRNA alone, but the best prediction occurred when protein and mRNA measurements agreed.

3. **Stark et. al. [18] compared the mRNA (RT-PCR) and protein levels (IHC) of apoptosis regulating genes p53, BCL-2 and BAX in breast cancer primary tumors and brain metastases.** The mRNA level of p53 was significantly lower in brain metastases than primary tumors but protein levels were only slightly lower (not significant). BCL-2 mRNA and protein expression were in good agreement; both significantly lower in brain metastases. BAX mRNA and protein levels were clearly discordant; mRNA levels were down in metastases while protein levels were higher.

4. CD20 antigen, a glycosylated phosphorylated protein expressed on the surface of B cells, is the target of the therapeutic monoclonal antibody rituximab. **Sarro et al. [19] quantified CD20 mRNA using RT-PCR and protein levels using quantitative immunoblots in chronic lymphocytic**

leukemia (CLL) cells from patients. They found that CD20 protein was decreased by about 60% in CLL cells versus healthy donors. However, CD20 mRNA levels were normal or near-normal in CLL cells and did not correlate with protein levels. The protein decrease is likely the reason for the lower effectiveness of rituximab against CLL compared to other B cell malignancies.

5. Matrix metalloproteinases (MMP) degrade extracellular matrix and have been implicated in tumor invasion and metastasis. **Lichtinghagen et al. [20] measured MMP-2 and MMP-9 levels along with tissue inhibitor of metalloproteinases (TIMP-1) in 17 patients with prostate cancer.** They measured mRNA using RT-PCR and protein levels using quantitative zymography in paired benign and malignant tissue samples. MMP-9 protein levels were higher in cancer tissue than in normal. However, there was no significant correlation between the mRNA and protein expression of MMP-2, MMP-9 and TIMP-1 in either cancerous or noncancerous tissue. Values of R_s for plots of protein versus mRNA from 34 tissue samples were 0.109 for MMP-2, 0.059 for MMP-9, and 0.077 for TIMP-1.

6. **Shebl et. al. [21] measured the secreted protein and cellular mRNA levels of 20 cytokines produced by peripheral blood mononuclear cells (PBMCs) from 26 women: 19 vaccinated with an GPV-16 VLP vaccine versus 7 placebo.** The cells were collected pre- and 2 month post-vaccination and cryopreserved until use. They were cultured for 72 hours in single wells *in vitro* with the VLP influenza A virus or baculovirus insect cell lysate (BAC) control. Supernatants and cell pellets were obtained from the same well. The cell free supernatants were tested in duplicate for cytokines using a 22-plex assay. Remaining supernatants and cell pellets were used for total RNA extractions and analyzed with Affymetrix Human Genome Focus Assay. Plots of protein versus mRNA were generated. Of the 20 cytokines analyzed, one, IFN-gamma, showed a very strong correlation with R_S of 0.90. Three cytokines (MIP1A, IP10 and TNF-alpha) showed correlations of 0.6 or higher. Five showed modest correlations of 0.4 to 0.59 (MCP1, IL-2, GM-CSF, IL-5 and RANTES). The remaining 11 cytokines showed weak or negative correlations. One conclusion of these authors was, "Investigators who use currently available expression array tools should be careful not to assume that mRNA expression changes identified by expression studies would necessarily reflect similar changes in corresponding protein levels."

Discussion

All mRNAs are not equal with regard to translation into proteins. Schwanhausser et al. [9] noted in their analysis of mouse NIH3T3 mouse fibroblasts that abundant housekeeping genes coding for ribosomal, glycolytic and TCA cycle proteins tended to have stable mRNAs and stable proteins. In contrast, transcription factors, signaling genes, chromatin modifying genes, and genes with cell-cycle-specific functions tended to have unstable mRNA and unstable protein. Thus, the most important and interesting regulators of cellular division and differentiation would be expected to have a poor correlation between mRNA and protein while mundane housekeeping proteins would probably have relatively good correlation.

Even mitochondrial house-keeping proteins may vary independently of mRNA changes. Marginantu et al showed that COLO 205 cancer cells treated with inhibitors of heat shock protein 90 (Hsp90) demonstrated immediate up-regulation of mitochondrial oxidative phosphorylation complex subunits without corresponding mRNA increases [22]. Total mitochondrial protein doubled after 48 hr treatment with Hsp90 inhibitors while expression levels of three non-mitochondrial proteins (proliferating cellular nuclear antigen, glyceraldehydes-3-phosphate dehydrogenase and tubulin) did not change. The Hsp90 mediated interaction of ubitiquitin ligases with their targets, and thus promoted mitochondrial protein

turnover via the ubitiquin-proteasome system. Hsp90 is in turn highly regulated [23] so this result most likely has practical ramifications.

Schwanhausser et. al. [9, 10] concluded that translational rate constants were the dominant factor in controlling protein levels and, if rate constants were known, the correlation coefficient would be boosted from 0.40 to 0.95. Wilheim et. al. in their quest to map the complete human proteome concurred that mRNA translation rates are key and suggested that the translation rate might be a constant for each transcript [13]. If so and if the translation rate constants were known, protein concentrations could be calculated from mRNA measurements as they showed in a back calculation. However, **MicroRNAs are known to simultaneously repress hundreds of genes by inhibiting mRNA translation** into protein and so modulate a great variety of mammalian cellular processes [24]. The human genome encodes over 1000 microRNAs [25]. The idea that the mRNA translation rate is a constant for each transcript regardless of cell type and microenvironment remains to be proven.

A multitude of post-translational mechanisms for controlling protein turnover and abundance have been well described. The human genome contains 630 E3 ubiquitin ligases and 553 proteases that regulate protein degradation. Dynamic post-translational phosphorylation is accomplished by 428 serine-threonine kinases, 90 tyrosine kinases, and 107 phosphatases. Phosphorylation status modulates mechanisms that control cell division and differentiation, dramatically affecting protein levels (11: Supplementary Sidebars 7.5, 14.10 and 16.8) Finally, cellular macro-processes such as the endocytosis-lysosome system are known to control protein half-lives. Given all the ways protein levels are controlled post-translationally, and given that mRNA translation is regulated by microRNAs in many cases, the 40% correlation with mRNA levels does not seem unreasonable.

Evolutionary selection for constant protein levels has recently been shown to be greater than selection for constant mRNA levels in lymphoblastoid cell lines from human, chimpanzee and rhesus macaque [26]. Dozens of genes were found that showed significant differences in mRNA expression between the species but little or no difference in protein expression. A mechanism for this kind of observation was suggested by Vogel and Marcotte [27] who wrote: "… steady-state abundances of proteins are determined by their functions and are based on, for example, matching stoichiometries between interacting proteins in the same physical complexes. Conservation of function between orthologues therefore implies conservation of protein abundances."… "it appears that mRNA levels of conserved genes diverge across time, but post-transcriptional, translational and protein-degradational regulation help to compensate for this drift and bring protein abundances back to evolutionary preferred levels."

Conclusions

The conclusion from the large amount of work summarized above is inescapable: mRNA levels cannot be used as surrogates for corresponding protein levels without verification.

A major research thrust over the past decade has been to find gene alleles or mutations whose expression correlates with a given disease. Such disease-associated genes or transcripts may serve as useful biomarkers, but are not necessarily predictive of disease mechanisms since proteins determine cell phenotypes, not DNA or RNA *per se*. A combination of genomic and proteomic tests would be expected to work better than either approach alone to clarify disease mechanisms.

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