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Stem Cells and the Translational Control of Differentiation: Following the Ribosome Footprints

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Abstract

Stem cells have been proposed as a promising source for cell therapy. Understanding the biological processes that commit stem cells to differentiate into a particular cell type is essential for the successful repair of injured tissue, and even for whole organogenesis. Cellular differentiation can be modeled as a network of regulatory circuits that direct various steps of gene expression and mediate the spatiotemporal control of a cell's proteome. In this mini-review, we discuss the current aspects of posttranscriptional regulation of gene expression in stem cells, with an emphasis on translational regulation. Several data supports the idea that a significant percentage of genes have their expression controlled at the translational level during stem cell commitment and differentiation. We focus on strategies using polysome and ribosome profiling to measure translational rates and to unravel the dynamics of this process.

Keywords: Stem cells; Translation; Differentiation; Polysome profiling; Ribosome profiling

Stem Cells and Translation

Stem cells proliferate, self-renew, and can differentiate into several tissue-specific lineages [1]. These characteristics make these cells ideal candidates for use in cell therapy [2,3]. However successful stem cell therapy requires that these cells be exclusively committed to the cell type that is needed. Stem cells respond to external stimuli that triggers the differentiation into a particular cell type [4]. Commitment involves the activation of a particular genetic program that is regulated at many stages during gene expression [5,6].

The identity and quantity of proteins that a cell produces under a particular set of conditions provides information about almost all cellular processes. Translation of mRNA into protein can be divided into three sub-processes - initiation, elongation, and termination. The initiation step includes all processes that precede the formation of the first peptide bond. This step starts with the binding of the eukaryotic initiation complex eIF4F (which contains eIF4E, eIF4A, eIF4B, and eIF4G) via the interaction of eIF4E to the 5 'cap of the mRNA in what is called the 'canonical cap dependent' process. After the mRNA has been unwound by eIF4F, the pre-initiation complex 43S (which contains the 40S ribosomal subunit, eIF3, and the ternary complex eIF2, GTP and Met-tRNAi) attaches to the 5'-proximal region of mRNA. This complex scans the 5'-UTR region to find the first AUG codon, and recruits the 60S ribosomal subunit to form the translationally competent 80S ribosome [7]. Initiation is followed by the elongation of the peptide chain, the main function of the ribosome, and by the termination step, which includes the release of newly synthesized protein and the dissociation of ribosomal subunits from the mRNA [8]. The initiation phase is the rate-limiting step in eukaryotes and is consequently the main target for translational control [9]. However, much structural and functional insight has been obtained for translation elongation and termination during the past few years, and has revealed a considerable role of these phases in translation control [10-12].

Regulation of translation plays a decisive role in a wide range of biological processes and is critical for maintaining homeostasis, cell proliferation, growth, and development. Deregulation of translation contributes to a number of human diseases including cancer [13-16]. Mechanisms that control translation can be roughly divided into two groups; global and transcript-specific control. Phosphorylation can modulate the activities of translation initiation factors, or the regulators that interact with them, enabling eukaryotic cells to regulate global rates of protein synthesis. During mRNA-specific control, the translation of a defined group of mRNAs is modulated without affecting general protein biosynthesis. This frequently occurs through the action of trans-acting RNA binding factors (RNA-binding proteins, miRNA, and tRNA fragments) which alter the translational fate of mRNAs [14,17,18].

Several studies have focused on the cellular transcriptome to understand the regulation of gene expression, with the assumption that mRNA abundance reflects the final concentration of proteins in the cell [19-21]. However, the half-life of an mRNA in the cell is governed by complex networks of RNA-protein interactions, from its transcription, to its processing, transport, storage and/or degradation, and finally translation [22-24]. Genome-scale analyses in eukaryotic cells comparing transcript and protein abundance have indicated that there is no direct correlation between mRNA abundance and protein synthesis, suggesting a high degree of posttranscriptional regulation in these cells. This hampers the classical transcriptome-based approach to analyze gene expression in stem cells. Indeed, protein abundance can be controlled and refined through the regulation of gene expression at various complementary stages [25].

For this reason, new methods have been developed over the past few years to compare the total amount of mRNA with the fraction of mRNA that is committed to translation. Analysis of polysome versus monosome and ribosome-free content (called polysome profiling) is a

well-established method to characterize and quantify the mRNA population associated with ribosomes and gives a readout of translation efficiency [21,26]. Ribosome profiling strategies are based on the deep sequencing of ribosome-protected mRNA fragments and enable the *in vivo* monitoring of translation with sub-codon resolution [27].

In this mini-review, we discuss the current aspects of posttranscriptional regulation of gene expression in stem cells, with an emphasis on translation and on strategies involving polysome and ribosome profiling.

Identifying genes that are translationally regulated during cellular differentiation

Cellular differentiation can be modeled as a network of regulatory circuits that direct various steps of gene expression and mediate the spatiotemporal control of a cell's proteome that in turn determines both cellular phenotype and plasticity [28].

The association of an mRNA with ribosomes is considered as a general measure of its translational activity [29]. Centrifugation of cytoplasmic contents in sucrose gradients separates polyribosome complexes from ribosome-free transcripts or inactive mRNP particles by density. Quantification of these mRNAs has been successfully used to obtain genome-wide information about translationally regulated mRNAs [29,30] (Figure 1A). This strategy has been used by several groups to investigate the post-transcriptional regulation of stem cell differentiation [31-37].

One study analyzed gene expression profiles during the differentiation of murine embryonic stem cells (ESCs) into embryoid bodies by integrating transcriptome analysis with a global assessment of ribosome loading [31]. The authors used sucrose gradient centrifugation combined with microarray analysis, also known as Translation State Array Analysis (TSAA) [30,38,39], to obtain a genome-scale view of the effect of translation on gene expression. Undifferentiated ESCs were found to be relatively polysome poor, as the result of inefficient loading of most transcripts onto ribosomes. Differentiation was accompanied by a global increase in both transcript abundance and the efficiency of mRNA translation. This study highlighted several vital genes that are exclusively regulated by translation during differentiation [31]. Human embryonic stem cells are isolated and characterized by surface marker expression. Kolle et al. [31] combined immune transcriptional profiling of human ESC lines with membrane-polysome TSAA to determine the genes encoding potential human ESC surface marker proteins [32]. This approach has been used extensively to profile transcripts encoding secreted or transmembrane proteins within a variety of cell model systems [40-43]. The assay separates mRNAs bound to actively translated, membrane-bound polysomes from cytosolic polysome-bound and non-translated mRNAs. A total of 88 genes that encode candidate cell surface markers of hESCs were identified with this approach, greatly expanding the number of protein antigens that can be used to isolate pluripotent ESCs [32]. Kolle et al. [32] also proposed astrategy to isolate mRNAs contained in the polysome-membrane fraction of hESCs and identified these RNAs by large scale sequencing [33]. They found that more than 1000 genes produce transcripts that contain long 5′ and/or extended 3′UTRs.Their analysis of membrane-polysome and cytosolic/ untranslated fractions also identified RNAs encoding peptides destined for secretion and the extracellular space [33].

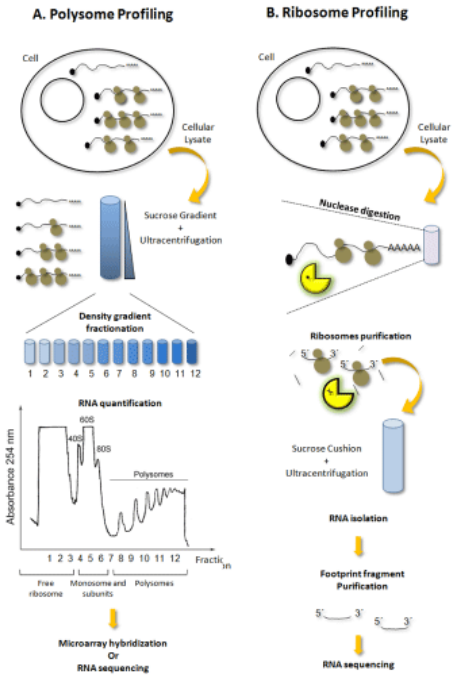

Figure 1: Steps of polysome and ribosome profiling (A) Polysome profiling. The cellular lysate is placed on a sucrose gradient allowing the separation of polysomal fractions. Actively translated polysome-bound mRNAs are denser than free mRNA and settle at the bottom of the sucrose gradient. Translationally inactive mRNAs that are not bound to ribosomes settle at the top of the gradient. The separation of the fractions is performed by Density Gradient Fractionation (ISCO) and afterwards RNA is quantified and isolated for microarray analysis or RNA sequencing. (B) Ribosome profiling (the deep sequencing of ribosome-protected mRNA fragments). The cellular lysate is treated with an RNA nuclease and ribosomes and associated mRNA footprints are purified by ultracentrifugation through a sucrose cushion. Protected mRNA fragments from single ribosomes are purified by PAGE and sequenced.

Similar findings have been reported for differentiation of adult stem cells. Parent and Beretta used polysome profiling to investigate translational control during hepatocytic differentiation of HepaRG liver progenitor cells [35]. They found that the vast majority of genes regulated during differentiation were contained in the polysome-bound RNA population and not in the total RNA population, suggesting a strong association between translational control and hepatocytic differentiation. Bates et al. [34] used a similar approach to analyze the translational regulation of genes in a model of B Cell differentiation. The authors used a streamlined version of traditional polysome profiling on a genomic scale during which mRNAs within sequential fractions of a linear sucrose gradient were differentially labeled and analyzed by DNA microarray [34]. This procedure, called Gradient Encoding, provides an accurate and reproducible ranking of the positions of mRNAs in the gradient, allowing sensitive detection of changes in the average number of ribosome per mRNA [44]. The authors found that during differentiation, major changes occurred in the posttranscriptional regulation of genes with critical roles in

transformation or differentiation. They also identified additional genes with potential roles in these processes based on particular changes in their translational regulation during differentiation.

An extensively studied model for adipogenesis *in vitro* is the mouse embryonic fibroblast cell line 3T3-L1 [45]. From m-Dornieden et al. [36] used TSAA to analyze changes to translational control at 6 hours after the induction of adipogenesis in 3T3-L1 pre adipocytes. The authors detected 43 translationally up-regulated mRNAs and two translationally down-regulated mRNAs [36]. Our group used polysome profiling of adult stem cells followed by RNA-seq analysis during the initial steps of adipogenesis to investigate how posttranscriptional regulation controls gene expression in human adipose stem cells (hASCs) [46]. RNA-seq analysis of the total mRNA fraction and the subpopulation of mRNAs associated with translating ribosomes showed that a significant percentage of mRNAs regulated during differentiation were post transcriptionally controlled. Our study identified 549 differentially expressed genes during initial steps of adipogenesis. Part of this regulation involved large changes in the length of untranslated regions (UTR), and the differential extension/reduction of the 3'UTR after the induction of differentiation. Large-scale sequencing allowed the identification of small RNAs, mRNAs, intrinsic regulatory elements in the mRNA sequence, and the length of the untranslated regions. Moreover, we showed that adult stem cells are committed to differentiation prior to phenotypic changes [46]. Polysome profiling is a simple and straightforward tool to analyze the flow of mRNAs between functionally distinct cell compartments, because these mRNA populations can be easily separated and isolated from a sample by centrifugation in a sucrose gradient. Its use in the study of translational regulation of stem cell commitment will help the understanding and identification of signals involved in the biology of these cells.

A ribosome footprint

Several studies have used polysome profiling to examine global translation in stem cells during various cellular processes, from proliferation to differentiation. Nevertheless, high molecular weight ribonucleoprotein complexes that are not committed to translation co-sediment with polysomes during this technique, which makes it difficult to separate mRNAs that are actually being translated [47,48].

However, the innovative ribosome profiling technique that was described by Ingolia and colleagues in 2009 has provided a detailed view of protein synthesis mechanisms from prokaryotes to mammals. This methodology relies on the fact that ribosomes protecta stretch (~30 nucleotides) of bound mRNA from nuclease digestion. This protected mRNA 'footprint' canthen be isolated and sequenced by deep-sequencing technologies (RNA-seq) (Figure 1B). Thus, it is possible to obtain the exact location of ribosomes on mRNA, as well as a detailed overview of all translation steps, including initiation, elongation and termination by this method. Ribosome profiling measures the number of ribosomes that are translating the mRNA in vivo, instead of the abundance of the transcript in the cell, providing measurements that closely correspond to protein abundance [27]. This methodology makes it possible to identify sequences that are actively translated, amongst a complex array of cellular transcripts. It also enables the monitoring of translation and maturation of nascent polypeptides in vivo, and the assessment of profiles of protein synthesis [48].

Ribosome profiling has emerged as a powerful technique to study several aspects of translation and is being used to unravel the mechanisms involved in the translational control of gene expression in stem cells. Pioneering work was carried out by Ingolia and co-workers in 2011 [47]. They obtained genome-wide maps of protein synthesis in mouse embryonic stem cells (mESCs) and detailed information about the kinetics and mechanism of translation elongation and coupled co-translational events [49]. The authors analyzed the cumulative distribution of footprinting counts at each codon, relative to the median density across the gene, and found thousands of pauses in the body of several transcripts. Analysis of the sequence around the pause site revealed a peptide motif associated with internal translational stalling that was not enriched in rare codons. In addition, they used a pulse-chase strategy to measure the rate of translation elongation and found that the kinetics of elongation do not depend on transcript length and protein abundance, even for transcripts that are translated at the ER surface. Furthermore, the authors suggest that translation speed does not depend on codon usage, which was consistent with the absence of pauses at rare codons. These results go against accepted biophysical models of translation, which state that elongating ribosomes translate each codon with a speed related to the features of the coding sequence and according to cellular factors, such as concentrations of elongation factors and tRNA molecules [50-52].

Recently, Dana and Tuller re-analyzed ribosomal profiles of mESCs measured by Ingolia and co-workers [47] and showed that translation elongation speed is affected by features such as the adaptation of codon to the tRNA pool, and local mRNA folding and charge. They also show that the translation elongation velocity tends to increase as translation progresses along the coding sequence.

Another intriguing result presented by Ingolia and co-workers [47] was the presence of several unannotated near-cognate initiation sites that drive the translation of upstream open reading frames (uORFs) in mESCs, consistent with the high rate of translation observed at many 5' UTRs. Translation of uORFs was lower in differentiating cells than in mESCs, indicating that the translation of uORFs is regulated and may be part of a major program of translational control. This finding prompted the authors to search for translated regions within some linc RNAs (long intergenic non coding RNAs) which have no conserved sequence with protein-coding potential. Mostputative linc RNAs were bound by ribosomes, raising the possibility that these transcripts encode small proteins. This is a striking observation; however, other findings strongly suggest that linc RNAs function as RNA molecules and not as translated proteins [53-55]. Thus, in a recent study the same researchers developed a metric termed ribosome release score (RRS) which analyzes the pattern of ribosome occupancy across different classes of RNA and distinguishes coding from non-coding transcripts [56]. The author's categorized lincRNAs with well-established non-coding RNAs, indicating that, in general, they do not encode functional proteins.

Ingolia and colleagues [27] examined changes in translation when proliferative; pluripotent mESCs underwent differentiation into embryoid bodies (EBs). The abundance of ribosomal proteins was much lower in EBs than in ESCs, due 3 to 4-fold difference in the translational efficiency of transcripts encoding ribosomal proteins between EBs and ESCs. Translation of uORFs also declined during differentiation, and the translation rate of 5' UTRs in differentiated cells was 25% lower than that of the CDS of individual transcripts with defined uORFs.

Adipogenic differentiation has been widely used by our group as a model to investigate the mechanisms of the posttranscriptional regulation of gene expression in hASCs. Polysome profiling

experiments showed extensive posttranscriptional regulation three days after the induction of adipocyte commitment [46]. Now, we have applied ribosomal profiling methodology to investigate differential gene expression of hASCs in the early steps of differentiation to obtain new insights into the mechanisms of translational control that may help to improve our limited understanding of stem cell differentiation. Preliminary data has confirmed extensive translational regulation during cell commitment, and shows that entire metabolic networks are regulated by translational mechanisms.

Ribosome profiling has also helped to characterize important proteins involved in mRNA metabolism in mESCs. One study used ribosome profiling to monitor translational efficiency after *Lin28a* knockdown [57]. LIN28 is a conserved RNA binding protein that is highly abundant in mESCs.LIN28acts as a suppressor of let-7 micro RNA biogenesis, however many lines of evidence suggest that LIN28 carries out additional functions. The ribosome occupancy of LIN28A-bound mRNAs tended to be higher in *Lin28a*-depleted cells than in control siRNA-treated cells, indicating that LIN28A targets mRNAs for translation repression.

A recent study investigated the implications of canonical and non-canonical Nonsense-Mediated mRNA Decay (NMD) on the decay of endogenous mRNAs in mESCs [58]. Messenger RNAs harboring upstream open reading frames (uORFs) may be susceptible to NMD, but only a fraction of uORF-containing mRNA is actually targeted by this pathway and the influence of uORFs on mRNA stability is poorly understood (reviewed by Hurt et al. 2013) [58]. Thus, the authors carried out ribosome profiling with UPF1-depleted and control-depleted mESCs. UPF1 is a conserved protein in eukaryotes that is essential for NMD. The density of footprinting reads was used to distinguish actively translated uORFs from non-translated uORFs. The depletion of UPF1 showed that actively translated uORFs-genes are normally targeted by NMD whereas non-translated uORFs-genes escape repression [58]. The authors concluded that NMD triggered by uORF translation is an important mechanism of the regulation of gene expression in mESCs.

Overall, translational regulation is the focus of intense study and is becoming increasingly appreciated as a central step of gene expression control. Polysome and ribosome profiling are powerful tools to analyze translational dynamics ona genome-wide scale and will enhance and improve our understanding of translational control during stem cell commitment.

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