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Downregulation of the protein synthesis machinery is a major regulatory event during early adipogenic differentiation of human adipose-derived stromal cells

Bruna H. Marcon [a], Fabíola B. Holetz [a], Guillermo Eastman [b], Ana Carolina Origa-Alves [a], Mariana Andrea Amorós [c], Alessandra Melo de Aguiar [a], Carmen K. Rebelatto [d], Paulo R.S. Brofman [d], Jose Sotelo-Silveira [b,e,*], Bruno Dallagiovanna [a,**]

[a] Instituto Carlos Chagas, Fiocruz-Paraná, Rua Professor Algacyr Munhoz Mader, 3775, Curitiba, PR 81350-010, Brazil
[b] Department of Genomics, Instituto de Investigaciones Biológicas Clemente Estable, Avenida Italia 3318, CP 11600 Montevideo, Uruguay
[c] Laboratory of Stem Cells, Institute of Biology and Experimental Medicine — National Council of Scientific and Technical Research (IByME-CONICET), Ciudad Autónoma de Buenos Aires, Argentina
[d] Núcleo de Tecnologia Celular, Pontificia Universidade Católica do Paraná, Rua Imaculada Conceição, 1155, Curitiba, PR 80215-901, Brazil
[e] Department of Cell and Molecular Biology, School of Sciences, Universidad de la República, Montevideo, Uruguay

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ABSTRACT

Commitment of adult stem cells involves the activation of specific gene networks regulated from transcription to protein synthesis. Here, we used ribosome profiling to identify mRNAs regulated at the translational level, through both differential association to polysomes and modulation of their translational rates. We observed that translational regulation during the differentiation of human adipose-derived stromal cells (hASCs, also known as adipose-derived mesenchymal stem cells), a subset of which are stem cells, to adipocytes was a major regulatory event. hASCs showed a significant reduction of whole protein synthesis after adipogenic induction and a downregulation of the expression and translational efficiency of ribosomal proteins. Additionally, focal adhesion and cytoskeletal proteins were downregulated at the translational level. This negative regulation of the essential biological functions of hASCs resulted in a reduction in cell size and the potential of hASCs to migrate. We analyzed whether the inactivation of key translation initiation factors was involved in this observed major repression of translation. We showed that there was an increase in the hypo phosphorylated forms of 4E-BP1, a negative regulator of translation, during early adipogenesis. Our results showed that extensive translational regulation occurred during the early stage of the adipogenic differentiation of hASCs.

1. Introduction

Stem cells (SCs) have the ability of both self-renewing and giving rise to new differentiated cells (Fuchs and Chen, 2013; Weissman, 2000). Adult SCs are multipotent cells found in different adult tissues. Human adipose-derived stromal cells (hASCs, also known as adipose-derived mesenchymal stem cells), a subset of which are stem cells, were first identified in 2001 (Zuk et al., 2002, 2001). Their ability to differentiate has been evaluated, and their potential use as a source for cell therapy has been studied (Kilroy et al., 2007; Mizuno et al., 2012; Rebelatto et al., 2008). However, for successful cell therapy, it is essential that these stem cells are committed to the cell type of interest. Commitment involves the activation of a well-defined genetic program regulated at multiple levels during gene expression. Understanding the biological processes that trigger differentiation into a specific cell type is essential for the successful repair of injured tissue or to undertake challenging goals such as whole organogenesis (Liu et al., 2007).

Most studies have focused on the characterization of the cellular transcriptome to understand gene expression regulation, assuming that the mRNA levels reflect the final concentration of proteins in the cell (Almalki and Agrawal, 2016; Jeong et al., 2007; Menssen et al., 2011). However, genome-scale analyses in eukaryotic cells comparing transcript and protein levels have indicated that there is a poor correlation between mRNA levels and protein synthesis (Tebaldi et al., 2012). Protein abundance can be controlled and refined through the regulation of gene expression at various complementary levels. The fate of mRNA in the cell is governed by complex networks of RNA-protein interactions, from transcription to translation and/or posttranslational modification (Balagopal and Parker, 2009; Pérez-Ortín et al., 2013; Vogel and Marcotte, 2012).

* Corresponding author.
** Correspondence to: B. Dallagiovanna, Instituto Carlos Chagas, Fiocruz-Paraná, Curitiba, Brazil.
E-mail addresses: jsotelosilveira@iibce.edu.uy (J. Sotelo-Silveira), bruno.dallagiovanna@fiocruz.br (B. Dallagiovanna).

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Regulation of translation is essential in a wide range of biological situations and is critical for maintaining homeostasis, growth and cell proliferation. Deregulation of translation contributes to a number of human diseases and types of cancer (Gkogkas and Sonenberg, 2013; Hershey et al., 2012; Pelletier et al., 2015). Translation can be regulated globally or at a transcript-specific level. Modulating the activities of translation initiation factors, or the regulators that interact with them by phosphorylation, often enables eukaryotic cells to regulate the global rate of protein synthesis. At the latter level, translation of a defined population of mRNAs occurs through the action of *trans*-acting RNA binding factors without affecting overall protein biosynthesis (Bhat et al., 2015; Hershey et al., 2012; Holcik, 2015).

Several lines of evidence from different model organisms suggest that stem cell self-renewal and differentiation are dependent on the control of protein synthesis by posttranscriptional mechanisms (Haston et al., 2009; Kolle et al., 2011; Sampath et al., 2008). The combination of quantitative proteomics with microarray analysis of mRNA levels in embryonic and primary hematopoietic stem cells showed a very low correlation between protein and mRNA expression during cell differentiation (Unwin and Whetton, 2006; Williamson et al., 2008). Although ribosome association with an mRNA is considered a general measure of its translational activity, in the past few years, new methods have been developed to compare the amount of total mRNA with the fraction of mRNA committed in translation. Polysome profiling and, more recently, ribosome profiling (RP) are related approaches that can quantify this association and have been successfully applied to the study of translational regulation in stem cells (Ingolia et al., 2011; Kuersten et al., 2013; Larsson et al., 2013).

Adipogenic differentiation has been widely used by our group as a model to investigate the mechanisms of posttranscriptional gene expression regulation in hASCs. Some of the transcriptional factors involved in the onset of adipogenesis, such as C/EBPβ, C/EBPγ and PPARγ, have already been described (Menssen et al., 2011); however, little is known about the translational regulatory mechanisms that participate in this process. We have previously used polysome profiling to study the commitment to adipogenesis in hASCs. We isolated both the total mRNA fraction and the subpopulation of mRNAs associated with translating ribosomes to understand the extent to which posttranscriptional regulation controlled gene expression in human adipose stem cells (hASCs). We observed that after 72 h of induction, the cells were already fully committed to differentiation. Our data showed that 60% of the genes that were differentially expressed after 72 h of induction showed some degree of posttranscriptional regulation and that this differential mRNA expression was associated with changes to the length of the 3'UTR (Spangenberg et al., 2013a, 2013b). However, we were not able to define how much of this regulation was specifically at the translational level.

In this study, we applied the RP methodology to gain new insights into the mechanisms of translational control that may help to further expand our limited understanding of adult stem cell differentiation. We observed extensive translational regulation during cell commitment, with entire metabolic networks being regulated at the translational level. After the induction of adipogenesis, hASCs showed a significant reduction in protein synthesis with a lower translational efficiency of ribosomal proteins. Our results revealed translational control as a key mechanism regulating the early steps of the adipogenic differentiation of hASCs.

2. Materials and methods

2.1. Subjects and cell culture

Human adipose stem cells (hASCs) were obtained from donors aged between 33 and 41 years, who were undergoing elective bariatric surgery and dermolipectomy procedures. Tissue collection and cell isolation were completed after donors had given informed consent, in accordance with the guidelines for research involving human subjects, and with the approval of the Ethics Committee of Fundação Oswaldo Cruz, Brazil (approval number 419/07). hASCs were isolated and characterized as previously described (Rebelatto et al., 2008). Briefly, 100 mL of adipose tissue were washed with sterile phosphate-buffered saline (PBS) (Gibco Invitrogen). A one-step digestion with 1 mg/mL type I colagenase was performed (30 mins; 37 °C) with permanent shaking, followed by filtration through 100 μm then 40 μm mesh filter (BD Biosciences). Cell suspension was centrifuged (10 min at 800 ×*g*; 8 °C) and erythrocytes contamination was removed by incubation with erythrocyte lysis buffer pH 7.3 (5 min). Cells obtained were washed and seeded at a density of 1×10^5 cells/cm^2 with DMEM-F12 (Gibco Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin and kept in humid incubator at 37 °C and 5% CO_2. After 24 h, non-adherent cells were removed and culture medium was changed twice a week. When culture reached 80–90% confluence, cells were washed with Ca2+ and Mg2+-free balanced salt solution (BSS-CMF), detached with 0.25% trypsin, replated as passage-1 cells and then cultivated with DMEM-F12 (Gibco Invitrogen) supplemented with 15% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. All tests and experiments were performed with cell cultures at passages 3 to 5. For adipogenic differentiation, cells (passage 5) were treated with culture medium supplemented with 200 μM indomethacin, 500 μM IBMX, 1 μg/mL insulin and 1 μM dexamethasone (all purchased from Sigma). The degree of adipogenic differentiation was assessed after 14 days of induction by fixing cells with 4% paraformaldehyde (20 min) and staining lipid droplets with 1 μg/mL Nile Red (30 mins, 4 °C), followed by cell counting as detected by fluorescence microscopy.

2.2. Ribosome profiling

The RP procedure was based on a previously described protocol (Ingolia et al., 2012, 2011) with slight modifications according to Smircich et al. (2015). Briefly, hASCs were incubated for 72 h with either adipogenic induction medium (induced cells, I) or maintenance medium (non-induced cells, NI). Then, cells were treated with 100 μg/mL cycloheximide (10 min; 37 °C), detached with trypsin, washed with PBS and incubated in lysis buffer (15 mM Tris HCl pH 7.4, 15 mM $MgCl_2$, 300 mM NaCl, 100 μg/mL cycloheximide, 1% Triton X-100, 20 μg/mL heparin) for 10 min on ice. Cell lysate was centrifuged (10 min at 12,000 ×*g*; 4 °C), and the supernatant was carefully collected, transferred into a new tube, incubated with benzonase (10 min; 25 °C) and RNase OUT™ (Invitrogen) to inhibit the action of nucleases. The digestion was loaded into an ultracentrifuge tube over 2 mL of a 1 M sucrose layer, and the ribosomes were pelleted by ultracentrifugation (2 h at 39,000 rpm, P40ST rotor, HIMAC CP80WX, HITACHI; 4 °C). The supernatant was removed, and RNA was extracted from the pellet with a mirVana™ kit, following the manufacturer's instructions. The RNA was submitted to electrophoresis, and the 30 nt region was excised. The RNA was then extracted and quantified.

2.3. Poly(A)+ mRNA isolation

Total RNA was isolated with TRIzol® Reagent (Invitrogen), according to the manufacturer's instructions. Next, poly(A) + RNA was extracted using a PolyATtract® mRNA Isolation Systems IV kit (Promega) following the manufacturer's instructions and then quantified.

2.4. Library preparation and sequencing

Samples of RP and poly(A) + RNA were prepared for sequencing using a SOLiD™ Total RNA-Seq Kit, following the manufacturer's instructions. Poly(A) + samples were first digested with RNAse III (10 min; 37 °C) to obtain 150–200 nt fragments, and RNA was purified.

As RP RNA had already been purified to isolate 30 nt fragments, this digestion step was not performed.

RP and poly(A) + samples were analyzed by deep sequencing on Life Technologies SOLiD4 equipment (high throughput sequencing facility RPT01G PDTIS/Carlos Chagas Institute, Fiocruz-Parana).

2.5. Data analysis

SOLiD raw data were imported into CLC Genomics Workbench 6.5 (https://www.qiagenbioinformatics.com/). In the CLC environment, we performed the following workflow: briefly, adapters were first trimmed from reads; then, quality ($p < 0.05$) and length trimming (transcriptome reads: length > 18 and translatome reads: 25 < length < 40) were performed as described by Smircich et al. (2015). Trimmed libraries were first mapped against a ribosomal RNA sequence database to remove rRNA reads, and unmapped reads were mapped against human mRNAs from the NCBI FTP site (ftp://ftp.ncbi.nlm.nih.gov/genomes/Homo_sapiens/RNA/). Tables with read counts were exported and used in R as input for the DESeq pipeline (Anders and Huber, 2010). Normalization and gene expression analysis were performed independently for transcriptome and translatome data sets. We considered a transcript to be differentially expressed if it had an absolute fold change value higher than 2 and an associated p-value < 0.05. Heat maps were also generated using the DESeq package. Genes showed in the Venn diagrams in Fig. S2 were obtained after filtering out genes with low count reads and high standard deviations (see also Fig. S2) with the following cut off: 300 normalized reads for the transcriptome and 150 for the translatome. All gene ontology analyses were performed using DAVID (Huang et al., 2009a, 2009b).

2.6. Data access

Data from this study is available in the Short Read Archive (SRA) of the NCBI data bank under the accession number PRJNA328260.

2.7. Methionine incorporation assay

When hASCs were approximately 80% confluent, we changed their media to either adipogenic induction media (I) or maintenance media (NI). At 0, 12, 24 and 72 h after the media change, the cells were washed twice and then incubated for 20 min with MEM-deficient media, free of L-methionine, to deplete endogenous methionine. Then, culture pulse-labeling medium, which consisted of the same MEM-deficient medium containing 0.2 mCi/mL [^{35}S]-methionine, was added to the cultures, which were incubated in a humid incubator at 37 °C and 5% CO_2 for 2 h. Cells were then washed twice with PBS and detached with 0.25% trypsin, and the labeled proteins were precipitated with trichloroacetic acid (TCA). For this, cells were deposited into glass fiber filters (Whatman G), air dried and incubated with 10% TCA for 30 min. Then, the filters were washed twice with 5% TCA and boiled at 95 °C for 15 min in the same solution. After washing again with 5% TCA, the filters were washed with 95% ethanol and air dried. Radioactivity was counted by liquid scintillation.

2.8. EdU incorporation assay

Prior to the assay, cells were treated for 72 h with either NI or I media. Then cells were incubated with 10 μM EdU (in NI or I media) for 24 h, detached with trypsin, fixed and stained with Click-iT® EdU Alexa Fluor® 647 Flow Cytometry Assay Kit (Molecular Probes, Thermo Fisher Scientific), following manufacturer's instructions. About 10,000 labeled cells were acquired with a FACSCanto II flow cytometer (BD Biosciences). The analysis was performed with Flow Jo software version 10.0.8r1.

2.9. Cell size measurements

For cell size measurements, cells were detached with trypsin, replated on chamber slides and allowed to adhere for 30 min. They were then analyzed under a Primo Vert microscope (Zeiss), and images from ten different fields were captured using Axio Vision software (Zeiss). For each condition, the cell diameter from 100 cells was measured using ImageJ software.

2.10. Migration assay

In vitro cell migration was performed using a 48-well modified Boyden Chamber (AP48, Neuro Probe, Inc.) equipped with an 8 μm pore size polycarbonate membrane (Neuro Probe, Inc.). Prior to the assay, cells were treated for 72 h with adipogenesis inducing factors and then serum deprived for 16 h. Briefly, cells were seeded in the upper chamber (1.2×10^4/50 μL DMEM-F12) and allowed to migrate toward the lower chamber. Either 10% FBS or DMEM-F12 (26 μL/well) was used as a chemoattractant or a negative control, respectively. After a 4h incubation at 37 °C in a 5% CO_2 atmosphere, the membrane was removed and fixed (4% PFA), and non-migrated cells were scraped. Migrated cells were stained with Hoechst 33342 (Sigma-Aldrich) and photographed using fluorescent-field microscopy. Images captured in 2 representative visual fields per well were analyzed using CellProfiler software (www.cellprofiller.com), and the mean number of cells/field was calculated.

2.11. Sucrose density gradient separation

Polysome fractions were obtained as previously described (Spangenberg et al., 2013a, 2013b), with slight modifications. Briefly, cells were treated with cycloheximide, detached and lysed as described for ribosome profiling. Then cell extract was loaded into a sucrose density gradient (10–50%) and submitted to ultracentrifugation (2 h at 39,000 rpm, P40ST rotor, HIMAC CP80WX, HITACHI; 4 °C). The sucrose gradient was fractioned using ISCO gradient fractionation system (ISCO Model 160 Gradient Former), connected to a UV detector (tuned to detect 275 nm absorbance) that recorded the polysome profile.

3. Results

3.1. Quantifying mRNA recruitment to polysomes by ribosome profiling

To study the early steps of differentiation and the events that led to cell commitment, we used hASCs submitted to adipogenesis. All experiments were performed with cells from at least three different donors. To verify the capacity of cells from different donors to undergo successful adipogenesis and to assess the degree of differentiation, hASCs were kept in induction medium for 14 days. After this period, cells from all donors were able to differentiate into fully mature adipocytes (Fig. S1A).

Because we wanted to study gene expression during the early steps of differentiation, we used hASCs cultured in maintenance medium (non-induced, NI) or treated with adipogenic induction medium for 72 h (induced, I) for RP analysis. Previous data has shown that after 72 h of treatment, hASCs have already triggered the differentiation pathway at the molecular level (Spangenberg et al., 2013a, 2013b) even though no phenotypic changes characteristic of adipogenesis were apparent (Fig. S1B).

NI and I cells were submitted to the RP protocol to isolate mRNA ribosome-protected fragments. On average, 60×10^6 reads were obtained for each poly(A) + sample and 360×10^6 for each RP sample. After size and quality trimming, the sequences obtained were mapped against an rRNA database to remove reads derived from ribosomal RNA. Seven and a half percent of the reads from poly(A) + samples and 75% of the reads from RP samples mapped against rRNA, likely because we did not perform a specific rRNA depletion protocol, as other groups have (Rooijers et al., 2013; Vasquez et al., 2014).

Sequences that did not match to the rRNA database were mapped against a human mRNA database. According to the cutoff specified in Material and Methods, poly(A) + transcriptome samples had 13,723 and 13,871 transcripts detected for the NI and I conditions, respectively. Of the RP samples, we identified 12,059 and 12,378 transcripts being translated for the NI and I conditions, respectively (Table S1). When comparing poly(A) + and RP data, we observed that the vast majority of identified genes were detected in both the transcriptome and translatome (Fig. S2C). Additionally, it is possible to see that RP fragments only match to annotated coding regions, while poly(A) + sequences map all over the transcript (Fig. S2D).

3.2. Ribosome profiling reveals extensive translational control during the early steps of hASC differentiation

To identify genes differentially regulated at the translational level after induction to adipogenesis, we compared mRNA levels at the transcriptome and translatome fractions (see scatter plots of Fig. 1A and B, respectively). Interestingly, we observed a higher number of regulated mRNAs in translation (2139 transcripts, Fig. 1B) than in transcription (1010 transcripts, Fig. 1A) at the following cut off: log2 (FC) > 1 or <− 1, p < 0.05. Differentially expressed genes can be found in Table S2. This suggests an important role of translational regulation during the beginning of the differentiation process. The net influence of translation on gene expression regulation was observed when the fold change of translation and transcription were compared through a scatter plot. There, an identifiable group of genes that are changing in translation but not in transcription can be observed (Fig. 1C). Quantitatively, the histograms of the distribution of fold change on the side of the scatter plot show a wider extension of regulation in the translation compartment. A high-resolution view of the translational changes from the NI to I state is depicted by a heat map of the differentially translated mRNAs (Fig. 1D). It is possible to observe both the up- and downregulation of mRNA translation of different subsets of mRNAs.

Exploring the KEGG database, it was possible to see that pathways involved in adipogenesis (such as the PPAR pathway) and lipid

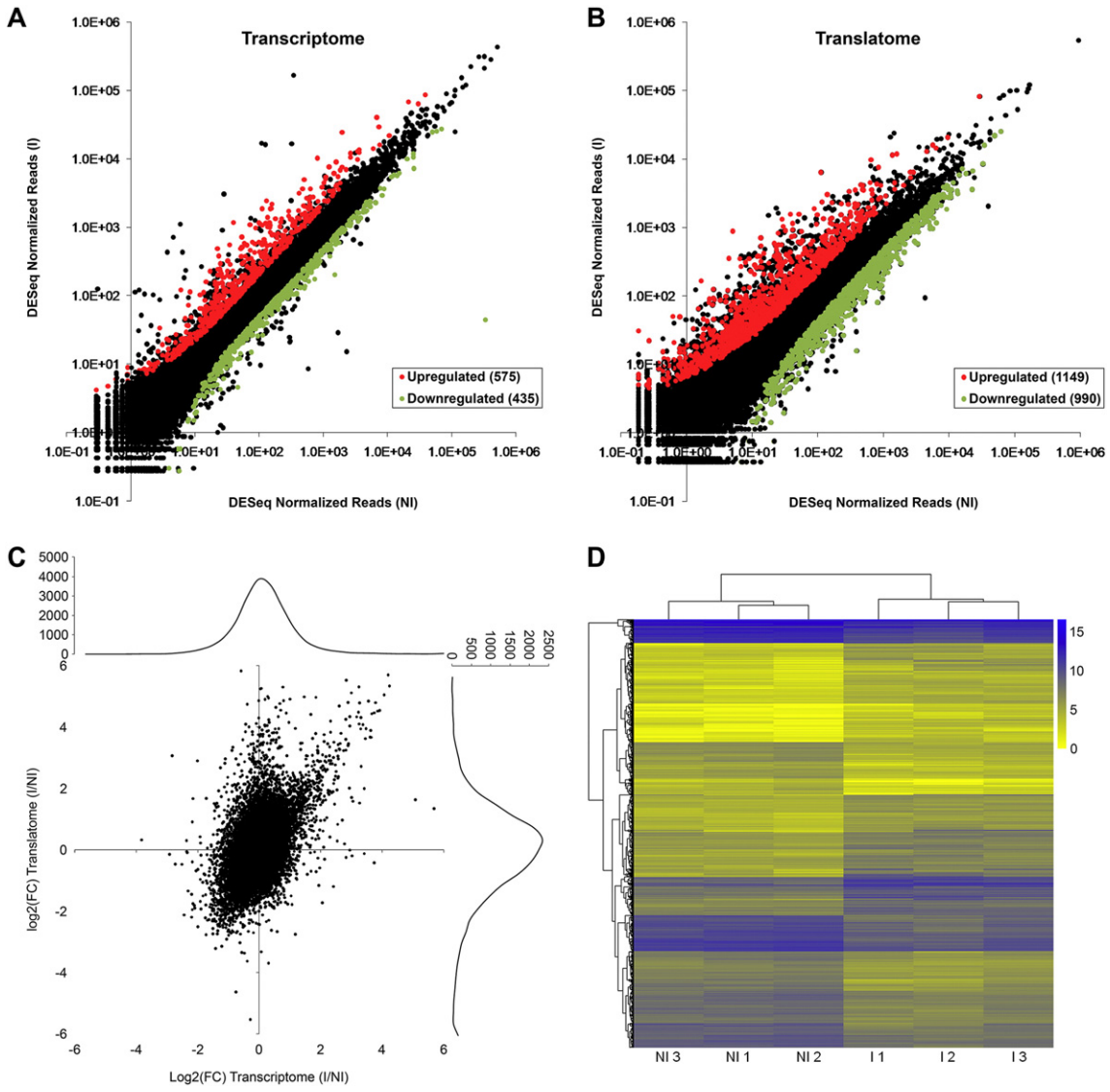

Fig. 1. Gene expression and regulation of translation during adipogenic induction. (A) Differential gene levels calculated by DESeq are shown for the transcriptome compartment. The number of DESeq normalized reads for each gene at each stage (NI and I) is plotted. We considered a gene to be differentially expressed if it had a fold change > 2 (up = red) or <−2 (down = green) and a p-value < 0.05. The number of differentially expressed genes is shown in the box in both panels. (B) Differential gene levels calculated by DESeq are shown for the translatome compartment. (C) Scatter plots where fold changes in translation are plotted against fold changes in transcription. In addition to the diagonal component observed, we observed a vertical component showing genes that were regulated exclusively at the translation level. On each axis, fold change histograms are included. (D) Heat map for differentially translated genes. The log2 values of DESeq normalized read counts were used.

biosynthesis were upregulated at both the transcription and translation levels (Table S3). This confirms previous results from our group showing that hASCs were already committed to differentiation after 72 h of induction.

Gene ontology (GO) enrichment analysis showed different profiles for poly(A) + and RP up and downregulated genes. Upregulated genes identified in the poly(A) + fraction were related to protein storage, catalytic, transporter, oxidoreductase and growth factor activities (Fig. 2A, Table S4). Upregulated genes identified in the RP samples coded for extracellular matrix proteins and proteins related to cell adhesion, receptor, catalytic and serine-type peptidase activities (Fig. 2A, Table S4).

In the poly(A) + fraction, genes that were downregulated were related to receptor, caspase, ligase and GTPase activator activities and steroid and clathrin binding proteins (Fig. 2B, Table S4). Downregulated gene categories in data from the translatome samples were related to structural protein constituents of the ribosome, structural constituents of the cytoskeleton, ligase activity and calcium ion binding proteins (Fig. 2B, Table S4).

We then calculated the translational efficiency (TE) of each gene and the fold change of TE (TEFC) between NI and I cells. Overall, 1128 transcripts had reduced translational efficiency when adipogenesis was induced ($\log2(TEFC) < -1$), while 1419 had an increased translational efficiency ($\log2(TEFC) > 1$) (Table S5).

Transcripts related to gap junctions, the Hedgehog pathway, cell adhesion molecules, dilated cardiomyopathy, viral myocarditis, axon guidance and the MAPK signaling pathway presented an increased TE (Fig. 2C, Table S6). Transcripts with a reduced TE included pathways related to ribosomes, pyrimidine metabolism, Parkinson's and Huntington's disease, oocyte meiosis and oxidative phosphorylation (Fig. 2D, Table S6).

3.3. hASCs show reduction in size, cell proliferation and decreased migratory activity during the early steps of adipogenesis

In the translatome assays, we observed a downregulation of genes related to actin cytoskeleton activity and focal adhesion proteins (Figs. S3A and S4A). Actin is a mechanical regulator of cell migration, and in hASCs, migration is also regulated through focal adhesion dynamics. The coordinated downregulation of these proteins could then be related to a reduction of cell migration ability. By immunofluorescence, no significant difference was observed in the organization of the β-actin cytoskeletons of NI and I cells, but there was a difference in the distribution pattern of phospho-FAK (Figs. S3B and S4B). Furthermore, the migration assay confirmed a significant reduction in the motility of cells induced to adipogenesis for 72 h compared to that of non-induced cells (Fig. 3A).

Changes in actin cytoskeleton have also been related to dynamic regulation of mitosis. Reorganization of the actin cytoskeleton contributes to cell cycle progression (Heng and Koh, 2010). Downregulation of actin expression could be related to lower proliferation rates of differentiating cells. We then performed EdU incorporation assay and found a reduction in cell proliferation after 72 h of adipogenic induction (Fig. 3B). We also measured the cell size of hASCs after 72 h of treatment with maintenance or induction medium. We found that there was an average reduction of almost 15% in cell size when cells were induced to differentiation (Fig. 3C).

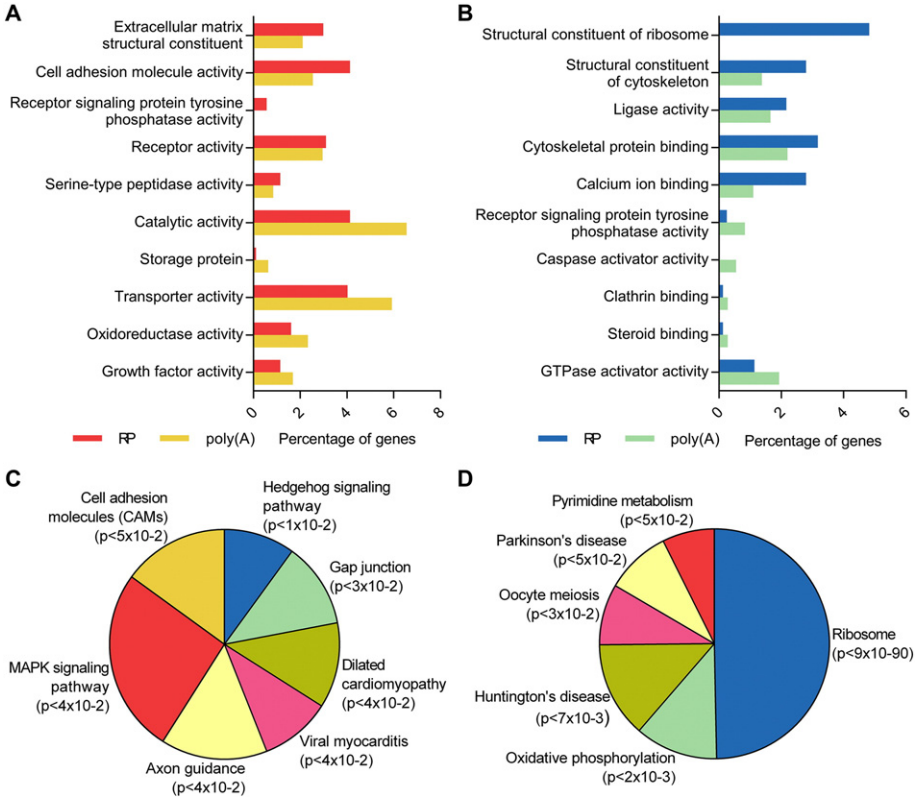

Fig. 2. GO and KEGG analysis of up- and downregulated genes at the beginning of the adipogenic differentiation of hASCs. (A) GO enrichment analysis of genes transcriptionally and translationally upregulated ($\log2$ (FC) > 1). p-Value < 0.05. (B) GO enrichment analysis of genes transcriptionally (poly A) and translationally (RP) downregulated ($\log2(FC) < -1$). p-Value < 0.05. (C) Enriched KEGG pathways found within genes with augmented translation efficiency ($\log2(TEFC) > 1$). p-Value < 0.05. (D) Enriched KEGG pathways found within genes with reduced translation efficiency ($\log2(TEFC) < -1$). p-Value < 0.05.

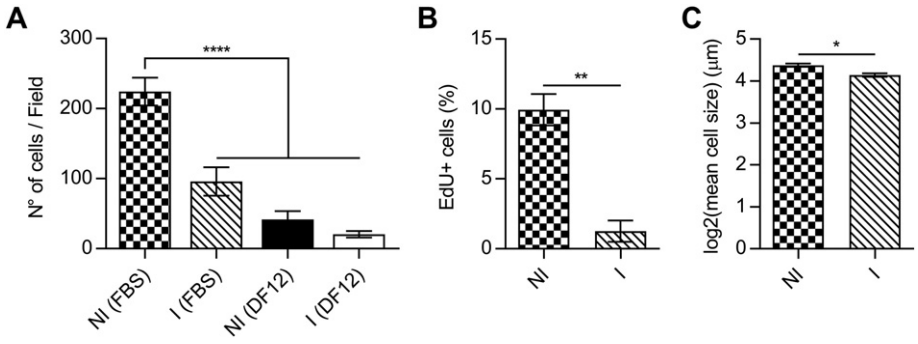

Fig. 3. Cell migration, proliferation and size are reduced during the early steps of adipogenesis. (A) hASCs were treated with maintenance or induction medium for 72 h and then submitted to a migration assay. The results represent the mean ± SEM of two independent experiments, performed with three donors each. ANOVA-Dunnett analysis: ****$p < 0.0001$. (B) hASCs were treated with maintenance or induction medium for 72 h and then submitted to proliferation assay. The results represent the mean ± SEM of three independent experiments. Student's *t*-test analysis. **$p < 0.01$. (C) The measurement of cell diameter using a microscope revealed a reduction of 14.68% in the mean cell diameter after adipogenesis was induced for 72 h. The results represent the mean ± SEM of three independent experiments. Student's *t*-test analysis: *$p < 0.05$.

3.4. Translational activity is reduced in early adipogenic differentiation

Interestingly, among the mRNAs with reduction in TE ($\log2(TEFC) < -1$), we found 81 transcripts coding for ribosomal proteins (Figs. 2D and 4A, Table S5). We arbitrarily selected the ribosomal RPS6 gene as an example, as similar patterns were observed for all ribosomal proteins. A comparison of the detailed mapping patterns of footprints from NI and I samples onto an RPS6 mRNA reference showed the decrease in the detected ribosomal protein translation efficiency ($\log2(TEFC) = -2.02$). Furthermore, although the number of detected reads in the RP samples was significantly reduced, there were no significant changes in the mapping pattern of ribosomal footprints between the NI and I conditions (Fig. 4B).

Next, we investigated if the variation in the TE of ribosomal proteins was related to changes in translational activity in cells during the beginning of the differentiation process. We used the [^{35}S]-methionine incorporation assay to measure the translational activity of cells cultured in maintenance medium and at different time points after the induction of adipogenesis (Fig. 4C). It was possible to see that in undifferentiated cells, there was an increase in methionine incorporation in the first 24 h after adding maintenance medium. [^{35}S]-methionine incorporation decreased as the cells reached confluence, and the nutrients in the culture medium were consumed. On the other hand, when induction medium was added, the incorporation of methionine did not increase when compared with the initial time point. Incorporation rates were clearly lower in induced cells than in undifferentiated cells at all tested time points (Fig. 4C). The reduction in the TE of ribosomal proteins (Figs. 2D and 4A, Table S5) and the reduction of protein synthesis activity (Fig. 4C) suggest that there is a strong translational downregulation during the early differentiation steps of hASCs into adipocytes.

We analyzed the polysomal profile of hASCs by centrifugation in sucrose density gradients. Though this was not quantitative, we could observe a clear reduction of the polysomal profile in hASCs after adipogenic induction (Fig. S5A and B).

Most regulation occurs at the initiation step of translation, where the eukaryotic small 40S ribosomal subunit is recruited to the 5′-terminal cap structure of mRNAs. The best-studied mechanism of this type of regulation involves the control of the availability of active eIF2 and eIF4F initiation complexes by reversible protein phosphorylation. In the case of the eIF4F complex, this occurs mainly by phosphorylation of the repressor eIF4E-binding proteins (4E–BPs) (Jackson et al., 2010).

No change in the phosphorylation profile of eIF2a after 24 and 72 h of treatment with adipogenic medium was observed by Western blot (Fig. 5A and B), suggesting that translation was not inhibited by this pathway. 4E-BP1 binds eIF4E and inhibits translation. 4E-BP1 binding to eIF4E is regulated by phosphorylation: hyper phosphorylation of 4E-BP1 reduces its ability to bind eIF4E, and translation activity increases. Conversely, 4E-BP1 hypo phosphorylation increases its binding activity and leads to an inhibition of translation (Nandagopal and Roux, 2015). There are at least four hierarchical phosphorylation sites in 4E-BP1 that are important for modulating its interaction with eIF4E. Phosphate groups are first added to Thr37 and Thr46, which are priming sites for the subsequent phosphorylation of Thr70, which in turn is required for the phosphorylation of Ser65 (Gingras et al., 2001; Proud, 2007).

By RP and by Western blot, we saw a reduction of 4E-BP1 expression after 72 h of treatment with induction medium ($\log2(FC) = -1.19$) (Table S2; Fig. 5D). Then, we analyzed the 4E-BP1 phosphorylation profile in hASCs 24 and 72 h after treatment with control or induction medium. After 24 h of cell induction, there was no significant change in the phosphorylation of Ser65 or Thr70. For Thr37/46, there was no significant change in the quantification. However, there was a clear change in the phosphorylation profile of these sites. Moreover, there was a significant increase in the amount of non-phosphorylated Thr46 in 4E-BP1 (Fig. 5C and Fig. S5C). After 72 h of induction for adipogenesis, there was a decrease in the amount of 4E–BP1. It was also detected a decrease in the overall phosphorylation of Ser65 and Thr70 (Fig. 5D). But this change was not observed when the phosphorylated protein levels were normalized to 4EBP1 total levels (Fig. S5D), suggesting that the reduction of these phosphorylated forms may be related to the decrease in the total amount of 4EBP1.

4. Discussion

Translational regulation mechanisms are considered key central players in the control of gene expression. Regulation occurs mainly at the formation of the translational initiation complex, in the assembly of translating polysomes or through the modulation of elongation. Compared to transcriptional regulation, translational control of functional mRNAs exerts rapid changes in cellular concentrations of the corresponding proteins and thus results in the triggering of gene networks involved in the commitment to differentiation and in the modulation of more permanent phenotypic changes in cell physiology (Sonenberg and Hinnebusch, 2009).

Translational control also plays a major role in embryonic stem cell (ESC) biology. These regulatory mechanisms are mediated by trans-acting factors in the form of miRNAs and RNA binding proteins that bind to cis-acting elements present in the mRNAs to regulate their cell and tissue expression in an mRNA-specific manner (Li and He, 2012; Shigunov and Dallagiovanna, 2015; Ye and Blelloch, 2014). In previous work, we used adipogenesis as a cell differentiation model to study the regulation of gene expression in hASCs. We observed that most differentially expressed genes were subject to posttranscriptional regulation (Spangenberg et al., 2013a, 2013b).

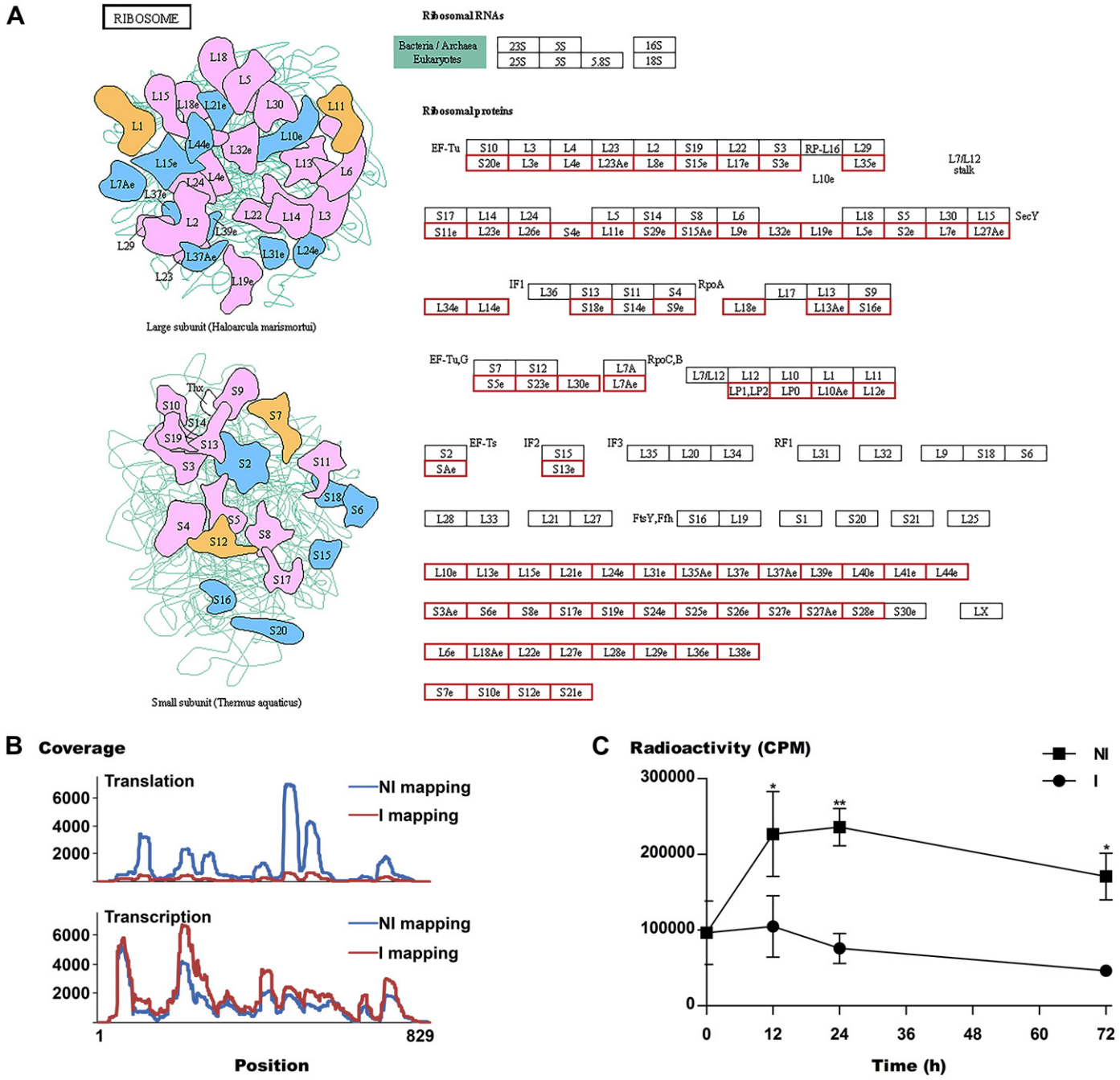

Fig. 4. Translational inhibition of ribosomal protein expression and reduction of translational activity in induced hASCs. (A) Ribosomal transcripts that have a reduction of translational efficiency (log2(TEFC) < −1) during the beginning of adipogenic differentiation (72 h) are marked in red (KEGG pathway). (B) poly(A) + and RP reads mapped onto an RPS6 transcript. After 72 h of adipogenesis induction, it was possible to see a reduction in the number of footprints in the RPS6 transcript, but the distribution pattern was similar. (C) Methionine incorporation assay showed a reduction in the translational activity after the induction of adipogenesis in hASCs (n = 3). The results represent the mean ± standard error of the mean (SEM) of three independent experiments. Student's *t*-test analysis: *p < 0.05 and **p < 0.01.

The identification of mRNAs associated with polysomes could provide us with a clearer idea of which genes are undergoing translation into proteins in differentiating stem cells and which of them are regulated at the translational level. RP involves the isolation of RNA fragments protected by ribosomes followed by next generation sequencing of the protected fragments ("footprints"). RP provides a global analysis of translation dynamics in a wide range of organisms, from yeast to mammals (Ingolia et al., 2009; Michel and Baranov, 2013).

We observed a high level of regulation at the translation level during early adipogenesis. A detailed analysis of the poly(A) + and translatome RNA fractions showed differences in the transcript composition of these two populations, regardless of donor origin. GO analysis revealed enrichment in different terms in the two populations with specific groups of related genes found to display differential expression in only the ribosome-bound fraction. These changes in expression levels included major differences in translational efficiency. We observed that for many genes, these changes in translational efficiency counterbalanced fluctuations in total mRNA levels.

Interestingly, we observed that essential biological processes during adipogenesis were controlled mostly at the translational level. Focal adhesion and actin cytoskeletal proteins have their translation rates downregulated at the onset of adipogenic differentiation. The potential

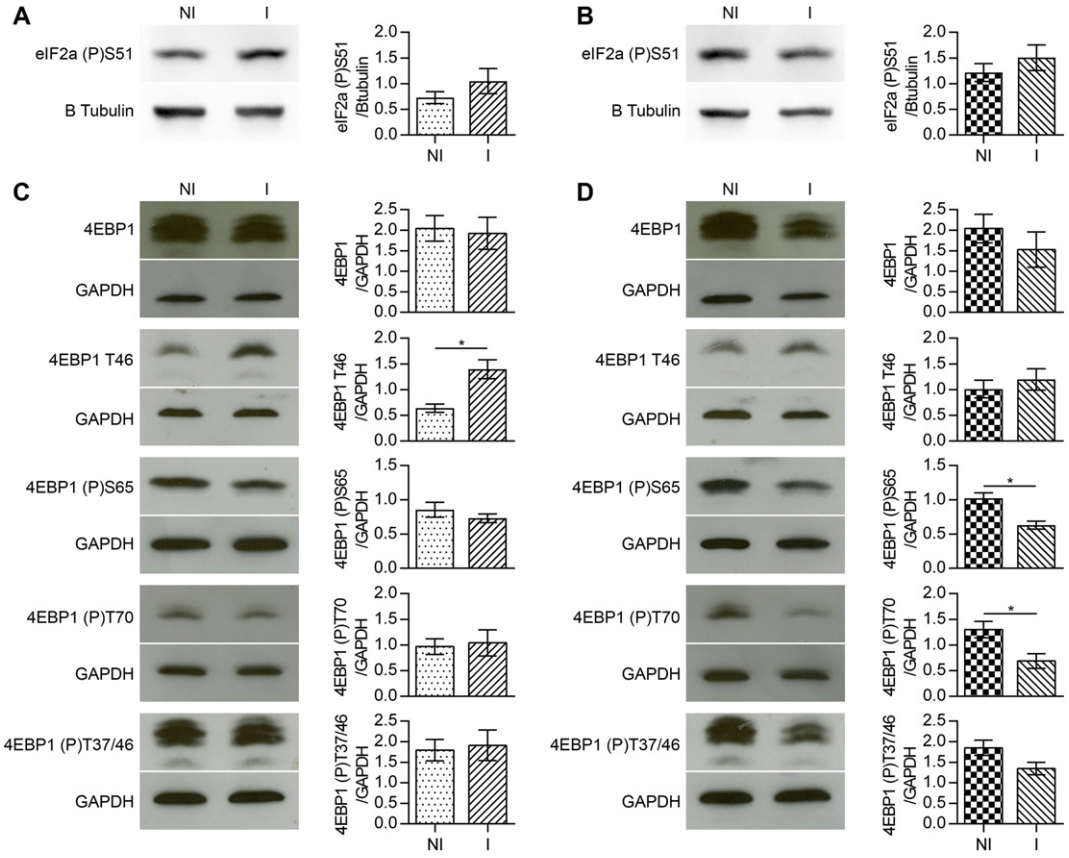

Fig. 5. Translational inhibition during the early steps of adipogenesis in hASCs. (A) The eIF2a phosphorylation profile of hASCs after 24 h of treatment with maintenance (NI) or induction (I) media. Representative image and quantification. The results represent the mean ± SEM of three independent experiments. Student's *t*-test analysis. (B) eIF2a phosphorylation profile of hASCs after 72 h of treatment with maintenance (NI) or induction (I) media. Representative image and quantification. The results represent the mean ± SEM of three independent experiments. Student's *t*-test analysis. (C) The 4E-BP1 phosphorylation profile of hASCs after 24 h of treatment with maintenance (NI) or induction (I) medium. Representative image and quantification. The results represent the mean ± SEM of three independent experiments. Student's *t*-test analysis: *$p < 0.05$. (D) The 4E-BP1 phosphorylation profile of hASCs after 72 h of treatment with maintenance (NI) or induction (I) media. Representative image and quantification. The results represent the mean ± SEM of three independent experiments. Student's *t*-test analysis: *$p < 0.05$.

use of hASCs in cell therapies depends, among other things, on their migratory capacity as a response to tissue damage. Once at the target site, adult stem cells need to stop migrating and graft to the injured region (Li and Jiang, 2011). Migration is driven both by the interaction between the actin cytoskeleton and focal adhesion complexes. A reduction in the number of focal adhesion complexes is related to higher rates of adipogenic and chondrogenic differentiation of hASCs (Mathieu and Loboa, 2012). Xu et al. (2014) also showed that inhibitors of focal adhesion kinase (FAK) and of cytoskeleton organization promoted adipogenesis. Here we show that this reduced expression, and thus lower migratory capacity of hASCs, is a result of a reduction of both translational efficiency and expression levels of these mRNAs. Cell migration also implies changes in cell morphology as a result of the rearrangements of the actin cytoskeleton (Gardel et al., 2010). Cells undergoing adipogenic differentiation have spherical morphology (Mathieu and Loboa, 2012). We showed that triggering adipogenesis involves drastic changes in cell size probably due to the combination of cytoskeleton rearrangements and lower overall protein translation.

Transcripts coding for ribosomal proteins were downregulated in their association to polysomes and thus showed a strong reduction in their translational efficiency. Negative regulation of ribosomal proteins has been reported during differentiation of different types of stem cells. In murine embryonic stem cells, Ingolia et al. (2011) observed strong repression of ribosomal protein translation as a response to differentiation into embryoid bodies. Similar results were reported during myogenic differentiation of murine C2C12 myoblasts (de Klerk et al., 2015). RP analysis showed downregulation of mRNAs coding for ribosomal proteins at the translational level.

We observed that this downregulation of ribosomal protein translation was associated with a clear reduction of protein synthesis in differentiating hASCs. Translational regulation is exerted mainly at the initiation step where there are two major control points. As already mentioned, regulation involves the modulation of the abundance and functionality of the eIF2 and eIF4F initiation complexes (Spilka et al., 2013).

A well-characterized mechanism of global translational control is mediated through phosphorylation of eukaryotic translation initiation factor 2 (eIF2). Phosphorylation of eIF2a results in a global arrest of translation usually associated with a stress response. Adult stem cells are present in all tissues in a quiescent state until they are stimulated to proliferate and migrate in response to tissue damage (Simons and Clevers, 2011). In muscle stem cells, eIF2a is phosphorylated in the quiescent satellite cell and is dephosphorylated when cells are activated to enter differentiation (Zismanov et al., 2016). However, no changes in the protein level or phosphorylation status of eIF2a were observed, suggesting that an alternative regulatory mechanism is responsible for the reduction in the rate of synthesis.

eIF4E function can also be modulated by changes in the abundance or activity of the protein. We observed no changes in the amount of eIF4F complex proteins. Mainly two signaling pathways, PI3K/Akt/mTOR and Ras/MAPK/Mnk, regulate the activity of eIF4E. The mTOR pathway phosphorylates the repressor 4E-BPs (eIF4E binding proteins), whereas the Mnk pathway phosphorylates eIF4E; both reactions allow for translation (Siddiqui and Sonenberg, 2015).

Our results showed that the amount of hypo phosphorylated 4E-BP1 is increased during the first days of adipogenic differentiation, which

correlates with a downregulation of protein synthesis. 4E-BP inhibits translation initiation by competing with eIF4G for a common binding site on eIF4E, and this inhibition depends on the protein phosphorylation level. In contrast, the hyper phosphorylated form dissociates from eIF4E, allowing for the interaction between eIF4G and eIF4E that leads to the initiation of translation (Pause et al., 1994).

As mentioned earlier in the text, multiple phosphorylation events are required to release 4E-BP1 from eIF4E, starting with the phosphorylation of Thr37/Thr46 and the subsequent phosphorylation of Thr70 and Ser65. Although phosphorylation of Thr37/Thr46 does not regulate the binding of 4E-BP1 to eIF4E directly, the Thr70 and Ser65 sites are adjacent to the eIF4E-binding motif and control binding to eIF4E (reviewed by Proud, 2007). As observed, there was a clear increase in the non-phosphorylated form of Thr46 and an important change in the phosphorylation profile of Thr37/Thr46 sites after treatment with inducing medium for 24 h. Additionally, we observed a significant decrease in the overall phosphorylation of Ser65 and Thr70 after 72 h of cell induction, that was mostly related to the decrease of 4EBP1 total level. These results suggest that translational downregulation may be achieved *via* inhibition of the eIF4F complex by eIF4E repression and that this mechanism is triggered in the first 24 h after cell induction.

The mechanistic target of Rapamycin (mTOR) is a master sensor of cellular homeostatic perturbations and plays crucial roles in cellular processes such as cell growth and proliferation. Negative control of ribosomal proteins translation has been directly linked to the mTOR pathway in different eukaryotic organisms from plants to mammalian cells (Iadevaia et al., 2012; Dong et al., 2015). Though our results point to a role of the mTOR pathway in the initial steps of adipogenesis, further work is needed to confirm our findings. No changes in the expression levels, association to polysomes or translational efficiency of mRNAs coding for mTOR related proteins was observed.

Other groups reported that mTOR activity is essential for adipogenesis, which appears as contradictory with our results. However, these observations were made in preadipocyte cells, which are already committed to adipocytes (Cho et al., 2004); or after mesenchymal stem cells reached confluence in culture, when cell growth and proliferation are already impaired (Yu et al., 2008). Alternatively, it has been shown that repression of the mTOR pathway is necessary to trigger adipogenesis. Overexpression of DEP domain containing mTOR-interacting protein (DEPTOR) promotes adipogenesis. It was suggested that DEPTOR activates adipogenesis by dampening mTORC1-mediated inhibition of insulin signaling (Laplante et al., 2012). Moreover, phospholipase D1 (PLD1) plays a negative role in adipogenic differentiation through activation of mTORC1 *via* displacement of DEPTOR from mTORC1 (Song and Yoon, 2016). The role of mTOR in adipogenesis appears to be more complex than expected and further work is needed to fully understand the mechanisms involved in this regulation.

Several other recent studies examining somatic stem cells in their tissular micro-environment have found interesting results regarding translational control, showing that quiescent stem cells have lower protein synthesis activity than progenitors, activated or differentiated stem cells (Blanco et al., 2016; Llorens-Bobadilla et al., 2015; Rodgers et al., 2014; Signer et al., 2014; Zismanov et al., 2016). Interestingly, Signer and collaborators have found that hematopoietic stem cells have lower rates of protein synthesis than other hematopoietic cells *in vivo* (Signer et al., 2014), and that this translational downregulation was related to 4E-BP1 and 4E-BP2 hypo phosphorylation (Signer et al., 2016). Moreover, Sanchez and collaborators have demonstrated that regulation of ribosome biogenesis and mTOR pathway is important for *Drosophila* germline stem cells differentiation (Sanchez et al., 2016). In our study, we found that, in culture, hASCs present a reduction in translational activity after adipogenic induction and this was accompanied by changes in the phosphorylation pattern of 4E-BP1. Although this is in apparent discrepancy with previous results, the experimental models and tissular micro-environments studied should be considered. Notably, by sucrose density gradient it was possible to see that the hASCs cultivated *in vitro* present a poor polysome profile before adipogenic induction, suggesting a low protein synthesis rate even before differentiation stimulus. Besides, in our study, we used subconfluent hASCs, an environment permissive to migration and proliferation, activities reduced when adipogenesis is triggered. Our results also showed that subconfluent hASCs used in our assays presented a different translational profile when compared to mesenchymal stem cells induced to adipogenic differentiation four days after confluency as in Yu et al., 2008. This reinforces the idea that culture conditions may influence hASCs features, and that this may not reflect *in vivo* scenario and transition between quiescent and activated states previously reported (Rodgers et al., 2014; Zismanov et al., 2016). Nevertheless, *ex vivo* studies are still important to understand cell biology. Moreover, regarding hASCs potential use for therapy and engraftment, the control of global translational activity in cell culture may be an important issue to fully take advantage of stem cell features.

Our results showed that extensive translational regulation occurred during the early stages of the adipogenic differentiation of hASCs. Major biological processes are regulated mainly at the translational level. Moreover, overall translation is reduced at the onset of adipogenesis. This downregulation could be, at least in part, the result of the inactivation of the eIF4F complex through 4E-BP repression.

Author contributions

BHM carried out the ribosome profiling, data mining and cell biology assays and drafted the manuscript. MAA performed the migration assays. FBH performed the ribosome profiling and RNA-Seq assays. GE carried out the bioinformatics analyses. CKR isolated the hASCs, and ACO performed the hASC differentiation assays. AMA helped with flow-cytometer analysis. PRSB coordinated the stem cell facility. JSS coordinated all bioinformatics analyses, designed ribosome profiling assays, data mining and helped to draft the manuscript. BD conceived and coordinated the study and wrote the manuscript. All authors read and approved the final manuscript.

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Disclosure declaration

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2017.10.027.

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