Analysis of post-transcriptional regulation using the FunREG method

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Abstract
An increasing number of arguments, including altered microRNA expression, support the idea that post-transcriptional deregulation participates in gene disturbances found in diseased tissues. To evaluate this hypothesis, we developed a method which facilitates post-transcriptional investigations in a wide range of human cells and experimental conditions. This method, called FunREG (functional, integrated and quantitative method to measure post-transcriptional regulation), connects lentiviral transduction with a fluorescent reporter system and quantitative PCR. Using FunREG, we efficiently measured post-transcriptional regulation mediated either by selected RNA sequences or regulatory factors (microRNAs), and then evaluated the contribution of mRNA decay and translation efficiency in the observed regulation. We demonstrated the existence of gene-specific post-transcriptional deregulation in liver tumour cells, and also reported a molecular link between a transcript variant abrogating HDAC6 (histone deacetylase 6) regulation by miR-433 and a rare familial genetic disease. Because FunREG is sensitive, quantitative and easy to use, many applications can be envisioned in fundamental and pathophysiological research.

Introduction
Post-transcriptional regulation is a set of biological processes controlling cell transcripts from their birth to their death (see [1,2] for more detailed reviews). This regulation plays a critical role in gene expression by providing the adequate amount of proteins for the functioning, growth, survival or contextual adaptability of cells. As a consequence, post-transcriptionally controlled genes are usually involved in transient and adaptable cellular processes such as signal transduction, cell proliferation, gene transcription, cell communication, metabolism or stimulus response [1,3]. Among the post-transcriptional processes, mRNA turnover and translation play a central role as these mechanisms govern directly, in a spatiotemporal manner, the quantity of proteins being produced by and distributed throughout the whole cell. The two key elements involved in these regulatory mechanisms are: (i) intrinsic cisARs (cis-acting RNA sequences); and (ii) their specific and specialized partners, called transRFs (trans-regulatory factors). Functional cisARs can be found throughout the mRNA sequence [4–6]. However the best-described ones, namely the ARE (AU-rich element) and the miRNA (microRNA) site, are mainly, but not exclusively, located in the 3′-UTR (untranslated region) (see the miRWalk website at http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk/) [7,8]. TransRFs are mostly represented by RBPs (RNA-binding proteins), small non-coding miRNAs and their cofactors [1,9,10]. RBPs belong to a very large family of proteins (an estimated 500 RBPs in yeast) bearing various types of RNA-binding domains, and involved in all steps of RNA metabolism and post-transcriptional control [2,11]. Post-translational modifications of RBPs (phosphorylation, methylation, etc.) provide another level of complexity in RNA-dependent mechanisms either by modulating the affinity of RBPs for their target RNAs or controlling their subcellular localization [12,13]. miRNAs are ~22-nt-long non-coding RNAs, generated nuclearily from either introns or specific primary transcripts (pri-miRNA) by a multistep process using the pre-mRNA splicing machinery or a dedicated microprocessor complex respectively [10]. Following their maturation by Dicer in the cytoplasm, miRNAs are incorporated into the RISC (RNA-induced silencing complex) and associated with the functional core protein Argonaute. Both RBPs and miRNAs control gene expression at a post-transcriptional level by modulating mRNA decay and/or translation efficiency. These controls are mainly linked to the polyadenylated and capped status of the target transcript [14,15].

Key words: functional, integrated and quantitative method to measure post-transcriptional regulation (FunREG), hepatocellular carcinoma, microRNA (miRNA), mRNA, post-transcriptional regulation; RNA-binding protein.

Abbreviations used: ARE, AU-rich element; cisAR, cis-acting RNA sequence; eGFP, enhanced green fluorescent protein; FunREG, functional, integrated and quantitative method to measure post-transcriptional regulation; HB, hepatoblastoma; HCC, hepatocellular carcinoma; HDAC6, histone deacetylase 6; miRNA, microRNA; qPCR, real-time quantitative PCR; RBP, RNA-binding protein; sRNA, small interfering RNA; TCN, transgene copy number per cell; transRF, trans-regulatory factor; UTR, untranslated region.

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Post-transcriptional regulation and pathology
Over the last decade, arguments underlying the importance of post-transcriptional deregulation in human diseases have accumulated [1,16,17]. Two major post-transcriptional...
defects have been reported in pathological cells: (i) loss of cis-ARFs by sequence mutation or deletion; and (ii) aberrant expression of trans-ARFs, exemplified by the HuR protein or miRNAs in cancerous tissue [1,10,18]. As RBPs can be phosphorylated or methylated, and miRNAs edited [10,12,13], it should be emphasized that such mechanisms may also influence gene expression and participate in ‘normal to pathological’ cell transition.

Sequence mutations or deletions of cis-ARFs are the result of genetic alterations or aberrations due to viral infections, chromosomal rearrangements, gains/losses of genetic information or, more rarely, to deficiencies in DNA surveillance and repair [1]. Genetic changes influencing mRNA decay or translation could also originate from normal events such as polymorphism [1]. In a collaborative work performed with our team, Benoît Arveiler and colleagues recently reported an example of such a genetic defect, which abrogates the regulation of HDAC6 (histone deacetylase 6) expression by miR-433 (see below and [19]). On the other hand, the expression of numerous trans-ARFs (either RBP or miRNA) is altered in diseased tissues [1,10,17]. Concerning RBPs, most studies have focused on ARE-BPs (ARE-binding proteins) in cancer, particularly on HuR [1,12,20]. But other RBPs, such as PTBP1 (polypyrimidine-tract-binding protein 1) in viral infection, can display altered expression in pathological tissues [21]. For miRNAs, numerous large-scale expression analyses have been performed in most diseased tissues, revealing specific marks or signatures which validate their use as biological markers in diagnosis [1,10,17,22].

**Post-transcriptional deregulation in liver cancer**

HCC (hepatocellular carcinoma) is the primary malignancy of the liver, and one of the most common and aggressive cancers worldwide (0.5–1 million deaths annually) [23,24]. Its childhood counterpart is HB (hepatoblastoma), a fetal-like liver cancer arising from undifferentiated liver stem cells. HCC is a heterogeneous tumour that develops on a diseased liver harbouring severe fibrosis or cirrhosis, themselves originating from hepatitis B or C virus infection, aflatoxin or alcohol consumption [25,26]. Its diagnosis is generally made at advanced stages of the disease when curative solutions, mostly based on surgery, can no longer be proposed, and patients are often affected with a recurrence of the disease. Several recent observations have argued in favour of an active participation of post-transcriptional mechanisms in HCC-associated gene alterations. First, Acevedo et al. [27] speculated that 40–50% of the changes in gene expression observed in HCC could originate from aberrant post-transcriptional regulation. Concomitantly, a comparative proteomic and transcriptomic profiling showed that the abundance of numerous proteins, whose expression varies between HCC and the adjacent non-tumour tissue, was poorly correlated with mRNA expression changes [28]. Finally, the altered expression of many trans-ARFs (either protein or miRNA) has been reported in HCC tissues [12,20,22,26]. However, important questions remain unanswered. How can it be demonstrated experimentally that post-transcriptional deregulation definitively participates in HCC-associated gene variations? How can this deregulation be studied in a laboratory context and the molecular factors involved be identified?

**Methods for measuring post-transcriptional regulation**

Many of the strategies summarized in Table 1 have been developed to study the molecular processes mediated either by cis-ARFs (located in mRNA coding or non-coding regions) or by trans-ARFs in mammalian cells. The methods first developed were devoted to the measurement of mRNA stability and the identification of cis-regulatory elements and trans-factors involved in mRNA decay, as exemplified by the transcriptional pulse assay [29]. Although these methods are very accurate, they have very limited application and have been used in only a small number of cell types (i.e. HeLa). Moreover, whereas some have profound indirect effects on mRNA decay (i.e. transcription inhibitors [30]), others require very specialized methodologies (i.e. cell-free extracts [31]) or time-consuming kinetics [6,29]. More recently, the dual-reporter system, either using fluorescent protein or luciferase, has allowed investigators to routinely and rapidly introduce post-transcriptional studies into a broader range of cells and conditions. The advantages and disadvantages of all of these methods in terms of the investigatory questioning and experimental context are summarized in Table 1. More detailed information can be found in the corresponding references. In the present paper, we focus on FunREG (functional, integrated and quantitative method to measure post-transcriptional regulation), a recently described method [32], and its potential applications in laboratories.

**FunREG method: principle and experimental pipeline**

FunREG was developed in order to determine whether or not the expression of particular genes (i.e. oncogenes) is post-transcriptionally altered in cancerous hepatic cells when compared with normal hepatocytes. However, we had to address several problems. First, the transgene reporter (post-transcriptionally regulated or not) had to be transferred into various human cell types, including primary human hepatocytes. Secondly, the interferences or biases due to transgene expression in host cells should be minimized as far as possible. Thirdly, the post-transcriptional mechanisms, mediated either by cis-ARFs or trans-ARFs, had to be measured in a quick, accurate and quantitative way, considering the limited amount of biological materials available from hepatocytes. Fourthly, to limit the use of laborious methodologies (i.e. kinetics in Table 1 and [6,29]), the measurements had to be achieved in ‘one time point’. Finally, the contribution of mRNA decay or translation...
<table>
<thead>
<tr>
<th>Method</th>
<th>Kinetics (K)/end-point (E)</th>
<th>Global post-transcriptional regulation</th>
<th>mRNA stability</th>
<th>Translation efficiency</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcription inhibitors</td>
<td>K</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>Easy and quick to perform; study of endogenous transcripts</td>
<td>Multiple non-specific effects; some inhibitors are cell-specific</td>
<td>[5,30]</td>
</tr>
<tr>
<td>Pulse-labelling and chasing assay</td>
<td>K</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>Study of endogenous transcripts; no transcriptional interference</td>
<td>Some assays use radioisotopes; possible low sensitivity</td>
<td>[5]</td>
</tr>
<tr>
<td>Transcriptional-pulse assay</td>
<td>K</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>Optimized for studying mRNA decay and poly(A) shortening</td>
<td>Transgene copy number unknown; possible saturation of the system and transcriptional interference</td>
<td>[5,29]</td>
</tr>
<tr>
<td>Mammalian cell-free extracts</td>
<td>K</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>Optimized for deciphering the molecular basis of mRNA decay and translation</td>
<td>In vitro study; lower decay rate than in vivo; specific experimental requirements; possible saturation of the system</td>
<td>[5,31]</td>
</tr>
<tr>
<td>Fluorescent- or light-dual-reporter system</td>
<td>E</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>Easy and quick to perform; commercial plasmid libraries</td>
<td>Transgene copy number unknown; possible saturation of the system and transcriptional interference, relative quantification</td>
<td>[34,36]</td>
</tr>
<tr>
<td>PCR-derived reporter system</td>
<td>E</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>mRNA stability and translation efficiency are both evaluated; easy and quick to perform</td>
<td>Transgene copy number unknown; possible saturation of the system and transcriptional interference; depends on DNA polymerase fidelity; relative quantification</td>
<td>[37]</td>
</tr>
<tr>
<td>FunREG method</td>
<td>E</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>mRNA stability and translation efficiency are both evaluated; stable transgene expression; no cell toxicity; large cellular spectrum including non-dividing cells; comparative cell analyses; transcription-independent</td>
<td>Not adaptable to the study of endogenous mRNAs; lentiviral facilities; relative quantification</td>
<td>[32]</td>
</tr>
</tbody>
</table>
efficiency in the observed post-transcriptional control had to be established, if possible at once.

As a lentivirus-production platform was available locally [33], and as we had already dealt with a fluorescent reporter system before [34], a strategy was elaborated based on the efficient delivery of transgenes by lentiviruses into a broad range of human cells, including non-dividing ones [33]. Having assessed in different cell types that the expression of an eGFP (enhanced green fluorescent protein) transgene was directly proportional to the number of integrated lentiviral transgene copies per cell (see Figure 2 in [32]), we speculated that any difference in the expression of a given transgene reporter subsequent to insertion of a definite regulatory sequence (i.e. 5’- or 3’-UTR) upstream or downstream of the open reading frame might exclusively originate from a post-transcriptional event. Therefore, by comparing, in a given cell type, the expression of an eGFP transgene bearing a regulatory RNA sequence (test transgene, Figure 1) and that of the same transgene devoid of it (reference transgene, as it serves as the reference for eGFP expression in this cell type, Figure 1), we should be able to measure the post-transcriptional control mediated by this sequence. It was on the basis of this idea that the FunREG experimental pipeline was built [32] (Figure 2). Following lentiviral infections,
three parameters were determined from each transduced cell population (reference and test). The amount of eGFP (P) was measured by flow cytometry using living cells. The TCN (transgene copy number per cell), and the amount of the corresponding mRNA (M) were measured by real-time qPCR (quantitative PCR) using genomic DNA and reverse-transcribed total RNA respectively [32] (Figure 2). Finally, these three parameters produced three ratios (Figure 2). In the transduced cell population, P/TCN represents the quantity of protein produced per transgene, M/TCN represents the quantity of mRNA produced per transgene (or the steady-state level of mRNA), and P/M represents the quantity of protein produced per mRNA. Now, in comparing, in a given cell type, the expression of the post-transcriptional-regulated eGFP test transgene with that of the reference, we assumed that P/TCN, M/TCN and P/M are indicative of the global post-transcriptional regulation, relative mRNA stability and relative translation efficiency respectively (see [32] for more details).

Validation of FunREG and applications in laboratory routine

To test FunREG function, we first measured post-transcriptional regulation mediated by a prototype ARE, or selected 3′-UTRs deriving from oncogenes in HCC cells (HuH7) by following the ‘cisARS-regulation’ pipeline [32] (Figure 2). Results clearly established the efficiency of FunREG for measuring post-transcriptional events mediated by the various regulatory sequences. As expected, results directly reported the contribution of either mRNA stability or translation efficiency in the observed regulation [32]. In a second set of experiments (depicted as the ‘transRF-regulation’ pipeline in Figure 2), FunREG was tested for its ability to sense regulation mediated by competent siRNA (small interfering RNA) and miRNAs, and determine the molecular process involved. The reporter expression of reference was given here by the cell population expressing the test transgene, and then transfected with an irrelevant small RNA (Figure 4 in [32]). Results revealed that the anti-eGFP siRNA not only induced mRNA decay, but also repressed translation. Although less potent than the siRNA, but in agreement with the literature [14,15], miR-98 and Let-7a efficiently reduced eGFP expression through c-myc 3′-UTR by destabilizing the mRNA, with limited effects on translation efficiency [35]. Finally, we assessed the capacity of FunREG to reveal post-transcriptional changes from one cell type to another in different pathological contexts (see [19,32]). We first compared cisARS function in cancerous and normal hepatocytes. In order to do so, HuH7, HepG2 (H1 cells) and normal hepatocytes were transduced by different eGFP reporter transgenes under the regulation of potent cisARSs. The FunREG pipeline was followed in each case, and the resulting ratios were compared. Results not only confirmed that cisARS-mediated regulation is cell-specific, but also showed that some oncogenic genes are post-transcriptionally deregulated in tumour cells. Results showed further that this deregulation is in accordance with an overexpression of the corresponding genes in HCC tissues, and that they concern variations of either mRNA stability or translational capacity. In a second study, FunREG was used to assess the functional consequence of a variant (c.281A>T [19]) found in HDAC6 3′-UTR. This variant was located in the miR-433 ‘seed’ sequence, and fully segregated with a familial dominant X-linked chondrodysplasia (disease caused by anomalies in chondrocyte differentiation and proliferation [19]). Using MG63 osteosarcoma cells, we demonstrated that this single base change was sufficient to completely abrogate the post-transcriptional regulation of HDAC6 by miR-433, therefore justifying the HDAC6 overexpression in tissues of affected patients [19].

Advantages of infectious lentiviruses for delivering a transgene into host cells

Experimental transduction using lentiviruses has many advantages over classical delivery methods using either chemical reagents or electroporation: it is quick, simple and minimally stressful for cells and for the biological process to be studied. Transduction has three more advantages over transfection: first, because the transgene integrates into the host cell genome, it is stably found in cell progenies. Secondly, as this integration occurs at random sites in the transduced cell population, the transgene expression becomes independent of its insertional position. Thirdly, the average number of integrated transgene copies per cell can be precisely defined, by using an adequate amount of infectious lentiviruses [32]. Therefore the investigator can design his experimentation in such a way that the mechanism being studied is not subjected to saturation or to non-specific biases. Finally, results obtained are guaranteed to be physiologically relevant. For these reasons, we generally work with cellular populations whose transduced cells contain an average of one transgene copy integrated per cell.

Concluding remarks and perspectives

As described above, FunREG is a unique, versatile and easy-to-handle method, allowing the measurement of post-transcriptional regulation mediated by cis- and trans-RFs in a broad range of experimental conditions and human cells, including non-dividing cells. FunREG connects transgene transfer efficiency and stable expression with laboratory routine methodologies for gene reporter detection (qPCR and flow cytometry). Results are achieved in a ‘one time point’ experiment, which avoids kinetic studies, but permits wide functional analyses and screening. No other methods so far provide, with such accuracy, both a global functional picture of a given post-transcriptional element or factor, and information about the contribution of mRNA stability or translation efficiency in the observed regulation. With the expanding utilization of lentiviruses in laboratories,
we believe that FunREG may be of particular help for numerous investigators familiar or not with post-transcriptional mechanisms. Initially, FunREG could be used as a prospective tool and help investigators to manage their experiments and future projects. As described in Figure 2 and [32], FunREG generates two transduced cell populations and necessitates a double technical handling of cells and materials. Soon, the FunREG pipeline will integrate a fluorescent dual-reporter system that overcomes the above constraints. In its new format, FunREG should be even more convenient for investigations regarding post-transcriptional analyses in laboratory routines, and should open the way to new areas of investigation in functional screening, combined -omics and human pathology.

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