RELATÓRIO FINAL

PROJECTO PTDC/BIM-MED/0352/2012

Objectivos do projecto

Functional upstream open reading frames (uORFs) are cis-acting regulatory elements of gene expression located upstream of the main initiation codon [usually, located in the 5' untranslated region (5'UTR)] that repress mRNA translation (protein synthesis) of the main ORF in physiological conditions. Under stress conditions, uORFs alleviate their repressive effect, as a response to the cellular environmental change (1). About half of the human mRNAs (mainly, transcripts encoding growth factors or hormones) present uORFs (1). However, for the vast majority of the cases, the principles by which uORFs participate in translational control are still poorly understood. The fact that mutations that introduce or disrupt a uORF can cause human diseases illustrates their role in translational regulation. Identifying when and how they function is essential to understand the etiology of many human disease-associated uORFs and establish their therapies.

In its classical hormonal role, human erythropoietin (EPO) is a glycoprotein synthesized and released mainly from the kidney, which has a key role in hematopoiesis (2). However, recent studies have revealed that EPO is a multifunctional molecule produced and utilized by many tissues (e.g. brain, heart and liver) that rapidly responds to different cell stress stimuli and tissue injuries. For example, EPO is upregulated in the brain and heart after injury and shows a tissue protective effect due to its antiapoptotic, anti-inflammatory, antioxidative and angiogenetic properties (3-7). Thus, it has the potential to be used as a therapeutic target/strategy for the treatment of several human disorders, including ischemic injuries in brain or heart (3-7). Understanding the EPO translational control mechanisms will be valuable in the determination of these therapies.

EPO is a 165 amino acid protein encoded by 1340 nucleotides (nts) transcript (NM_000799) that presents a 5'UTR with 181 nts containing a uORF with 14 codons that terminates 22 nts upstream of the EPO initiation codon. However, the role of this uORF is unknown. The aim of this project was to study the role of the EPO uORF in its translational control. More specifically, we aimed to investigate:

1. The role of the EPO uORF on the translational efficiency of the main ORF in different cell lines: REPC (kidney) (8), H9c2 (embryonic myocardium), HEK293 (embryonic kidney), C6 (neural), HepG2 (liver) and HeLa (cervix) cells, under physiological conditions;

2. The mechanism by which the uORF represses translation of the main ORF, in these cells at physiological conditions. For that, we tested: (a) if translational inhibition by the uORF is peptide sequence-dependent; (b) if the uORF induces nonsense-mediated mRNA decay (NMD) and down-regulates EPO expression; (c) if translational inhibition by the uORF involves ribosome stalling; (d) the mechanism of translation initiation at the EPO main AUG codon;
3. The mechanism by which translational inhibition mediated by the EPO uORF is overridden in response to several cell stress stimuli;
4. The presence of the uORF-encoded 14 aminoacids peptide in the cell lines and in vivo in human serum samples;
5. The functional and biological relevance of the EPO uORF translational regulation in mouse models of myocardial ischemia;
6. The relevance as therapeutic target or diagnostic/prognostic biomarker, in cardiac diseases, of protein(s) identified as being involved in the derepression of the EPO uORF in response to myocardial ischemia.

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Breve descrição das atividades desenvolvidas bem como dos desvios ocorridos durante a execução do projecto

Results from tasks 1, 2 and 3, allowed us to conclude that expression of EPO is regulated at the translational level by the 14 codons-uORF in HEK293, HepG2, HeLa, SW1088 (a neural cell line used instead of C6 cells), H9c2 and C2C12 (muscle) cell lines. For that, reporter constructs, containing the normal or mutant EPO 5'UTR sequence fused to the Firefly luciferase reporter cistron, were tested in all referred cell lines. Luciferase activity was measured by luminometry assays and the corresponding mRNA levels quantified by real-time RT-PCR. Then, luciferase activity was normalized to the corresponding mRNA levels. Results revealed that the EPO uORF represses translation of the main ORF in all cell lines, and synthesis of EPO protein mainly occurs by reinitiation after translation of the uORF. Moreover, we have shown that EPO uORF

functions in a peptide-independent manner, and does not induce ribosome stalling or NMD.

In addition, we observed that the EPO uORF responds to hypoxia (cobalt chloride cellular treatment) but not to nutrient starvation, specifically in HeLa cells but not in HEK293 or HepG2 cells. This EPO uORF-mediated translational repression is significantly released, through a mechanism that involves processive scanning of ribosomes from the 5' end of the EPO transcript and enhanced ribosome bypass of the uORF, increasing the number of ribosomes that initiate translation at the downstream main AUG codon. In these cells, hypoxia induces the phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2a) concomitantly with a significant increase of EPO protein synthesis.

Also, we observed that in C2C12 cells under hypoxia, or ischemia (iodoacetic acid cellular treatment), the EPO uORF-mediated translational repression is released; however, this translational derepression seems to occur through a mechanism independent of eIF2a phosphorylation. In cardiac H9c2 cells, the EPO uORF repression is specifically released in response to hypoxia. The molecular basis underlying these different responses is still unclear.

These findings provide a framework for understanding that production of high levels of EPO in response to cellular stress involves regulation at the translational level; however, this EPO uORF-mediated translational regulation seems to occur through different tissue-specific mechanisms.

Regarding task 5, transgenic mice were obtained during the last year of the project. These mice carry the luciferase reporter gene under the control of the active or inactive human EPO uORF. We are now genotyping the corresponding transgenic mice progeny to perform bioluminescence assays, in order to test whether EPO uORF mediates reporter luciferase upregulation after heart ischemia injury. Task 6 will be performed based on the results we will get in the experiments corresponding to task 5.

During last year of the project, we also developed 2 additional tasks:

1. Analysis of the cellular function of the EPO uORF-encoded peptide;
2. Analysis of the role of the translation initiation factor 3 (eIF3) in the mechanism of translation reinitiation using the EPO uORF reporter construct as experimental model.

To analyze the cellular function of the EPO uORF-encoded peptide, we cloned reporter constructs in which we inserted the FLAG tag into the C-terminus of the intact or inactivated EPO uORF. We are still performing immunofluorescence assays to localize the EPO uORF-encoded peptide in the cell.

To analyze the role of the translation initiation factor 3 (eIF3) in the mechanism of translation reinitiation, we knocked down the expression of each one of the 13 eIF3

subunits (a to m subunits) in HeLa cells and analyzed the consequences in translation reinitiation efficiency after translation of the EPO uORF. Results show that eIF3e, f, and h subunits are necessary for the mechanism of translation reinitiation.

Desvios ocorridos durante a execução do projeto

According to the suggestion of the Evaluation Panel, task 4 (above indicated) was not performed.

REPC cells (8) were not used as they were contaminated in the lab where they were established – in fact, the paper describing this cell line has been retracted [Retraction in: Blood. 2014 May 22;123(21):3365].

SW1088 cells (brain astrocytoma) were used instead C6 (glioma) cells.

An additional cell line was used: C2C12 cells (mouse muscle myoblasts).

Furthermore, we added and developed two additional tasks to this project:

1. Analysis of the cellular function of the EPO uORF-encoded peptide;
2. Analysis of the role of the translation initiation factor 3 (eIF3) in the mechanism of translation reinitiation using the EPO uORF reporter construct as experimental model.

Until the end of the project, it was not possible to analyze the obtained transgenic mice, as we need to wait for the transgenic mice progeny that only now is available for genotyping and further usage.

Regarding the duration of the project, FCT gave permission to develop the project for more 7 months, and thus the project closed by the end of 2015. Also, the budget was reprogrammed to cover these additional months.

Objectivos atingidos

In this project, we got the following main results:

1. The human EPO uORF represses translation of the downstream main ORF in HeLa, HEK293, HepG2, H9c2, SW1088 and C2C12 cells, under physiological conditions;
2. The mechanism by which EPO uORF represses translation of the downstream main ORF, in these cells at physiological conditions is not peptide sequence-dependent and does not involve induction of NMD or ribosome stalling;
3. EPO protein synthesis occurs mainly through the mechanism of translation reinitiation;
4. The mechanism of translation reinitiation depends on the following eIF3 initiation factor subunits: eIF3e, f, and h.

5. The EPO uORF specifically responds to some cellular stresses in a tissue specific manner: in response to hypoxia, it specifically derepresses translation of the main ORF in HeLa, H9c2 and C2C12 cells; however, the mechanism by which this translational derepression occurs in HeLa cells is different from what occurs in C2C12 or in H9c2 cells. In addition, EPO uORF responds to chemical ischemia in SW1088 cells and C2C12 cells, by derepressing translation of the main ORF; the mechanism involved in this translational derepression is still unknown.

Part of these results was published in the RNA Journal. The other results are part of two manuscripts in preparation (in attachment).

Realização Financeira (justificação sumária dos desvios ocorridos durante a execução do projecto)

This project was developed during 31 months, instead of 24 months originally planned (from June 2013 until December 2015); also, the budget was reprogrammed to cover these additional months:

1. The budget for consultants was reprogrammed to human resources, since the meetings with the project's consultant, Prof. Stephen Liebhaber from University of Pennsylvania, USA, were via SKYPE);
2. The human resources budget corresponding to last two months of BI fellowship was reprogrammed to service procurement and acquisitions because the BI fellow left.

Publicações obtidas durante o projecto

Publicações em revistas científicas:

Barbosa C, Romão L (2014)."Translation of the human erythropoietin transcript is regulated by a upstream open reading frame in response to hypoxia." RNA 20(5):594-608. doi: 10.1261/rna.040915.113. Epub 2014 Mar 19, (aceite em Janeiro de 2014) URL: http://rnajournal.cshlp.org/content/20/5/594.full.pdf html

Manuscritos em preparação:

Onofre C, Barbosa C, Romão L (2016). Translation of the human erythropoietin transcript is regulated by an upstream open reading frame in response to stress in cardiac and muscle tissue.

Barbosa C, Romão L (2016). The role of the erythropoietin upstream open reading frame in the human neuronal tissue.

Capítulos de livro:

Barbosa C, Onofre C, Romão L (2014). Upstream open reading frames and human genetic disease. In: eLS. John Wiley & Sons, Ltd: Chichester. (By invitation). DOI: 10.1002/9780470015902.a0025714

Teses:

Barbosa C (2013). Translational control by an upstream open reading frame in the human erythropoietin transcript. Doutoramento em Biologia (Biologia Molecular) da Faculdade de Ciências da Universidade de Lisboa. Bolsa FCT SFRH/BD/63581/2009.

Abstracts de comunicações científicas:

Barbosa C, Romão L (2013). Translation of the human erythropoietin transcript is regulated by an upstream open reading frame in response to hypoxia. The 18th annual meeting of the RNA Society, 11-16 June, Davos, Switzerland

Onofre C, Barbosa C, Romão L (2014). Translational control of the human erythropoietin via an upstream open reading frame in cardiac tissue. The 19th annual meeting of the RNA Society, 3-8 June, Québec City, Canada

Romão L, Peixeiro I, Onofre C, Barbosa C, Teixeira A (2014). The interplay between mRNA translation and nonsense-mediated decay in transcripts with short open reading frames. 9th FASEB meeting on "Post-transcriptional control of gene expression: Mechanisms of mRNA degradation. 6-11 July, Big Sky, Montana, USA

Onofre C, Barbosa C, Romão L (2014). Translational control of the human erythropoietin via an upstream open reading frame in cardiac tissue. XVIII Reunião Anual da Sociedade Portuguesa de Genética Humana. 19-21 Novembro, Lisboa, Portugal

Onofre C, Menezes J, Peixeiro I, Costa N, Barbosa C, Romão L (2015). How mRNA translation is involved in modulating nonsense-mediated decay in transcripts with short open reading frames. RNP and Disease Conference. 14-17 October, Marrakesh, Morocco

Onofre C, Barbosa C, Romão L (2015). Translational control of the human erythropoietin via an upstream open reading frame in cardiac tissue. EMBO/EMBL Symposium on Protein Synthesis and Translational Control. 9-13 September, Heidelberg, Germany

ATTACHMENT

(Manuscripts in preparation)

The role of the erythropoietin upstream open reading frame in the human neuronal tissue

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Running head: EPO translational control in neuronal cells

Key words: erythropoietin (EPO); ischemia; translational regulation; upstream open reading frame (uORF); brain; hypoxia

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ABSTRACT

Beyond its role in erythropoiesis, erythropoietin (EPO) plays several other non-hematopoietic roles as a consequence of its expression in other tissues, such as the brain, where it acts as a neuroprotector. EPO expression is tightly regulated in order to maintain its correct expression in response to stress conditions. *EPO* transcript contains an upstream open reading frame (uORF) of 14 codons. We have previously shown that *EPO* uORF is functional in HepG2, HEK293 and HeLa cells, and that its repression is released in HeLa cells under hypoxia, proving the importance of EPO expression in response to stress. Here, we show that *EPO* uORF is also functional in the brain, using SW1088 cells. Our data demonstrate that the uORF AUG is recognized by the preinitiation complex, thus inhibiting the recognition of the main AUG. Yet, some ribosomes bypass the uAUG and others reinitiate after uORF translation, allowing the production of the EPO protein. Moreover, we prove that *EPO* uORF functions in a peptide-independent manner and independent of *EPO* 3' untranslated region (3'UTR). In addition, we observe a uORF-dependent induction of *EPO* translation under chemical ischemia. The molecular basis underlying this process is still unclear, but these findings propose a specific regulation of EPO expression in the neuronal tissue.

INTRODUCTION

Translational control comprises a variety of mechanisms responsible for maintenance of homeostasis and for an accurate response of organisms to internal and external stimuli. Regulation of gene expression at this level accounts for quick and reversible changes on global translation or on a subset of selectively targeted messenger RNAs (mRNAs).

mRNAs have, both in the 5' leader sequence and in 3' untranslated region (UTR), evolutionary conserved features that may influence their translational rate and even their stability. Examples are regulatory upstream open reading frames (uORFs), internal ribosomal entry sites (IRESs) and binding sites for proteins or microRNAs [1].

uORFs are regulatory *cis*-acting elements present in the 5' leader sequence of a transcript. These elements are common to genes that need to be tightly regulated, including oncogenes and genes involved in the control of cellular growth and differentiation [2,3]. Although their presence throughout the genome has been demonstrated, their prevalence has been difficult to calculate [4]. The most recent studies estimate that about 49% of the human transcripts contain at least one uORF [5].

In order to be functional, a uORF has to be recognized and translated. Its AUG is recognized by the scanning 40S ribosomal subunit and associated initiation factors depending on its context [6]. The optimal context is GCC(A/G)CCAUGG, being the -3 and +4 the most important. An AUG in this context is putatively recognized by all the

ribosomes that encountered it. However, differences on this sequence can modulate the strength of the AUG context resulting in the bypass of some or all preinitiation ribosomal complexes altering the translational efficiency of the uORF [7]. This mechanism is called leaky scanning and is also affected by the AUG proximity to the cap site and the presence of nearby secondary structures. Additionally, some uORFs promote ribosome stalling during elongation or termination phases, creating a blockade to additional ribosome scanning [8,9]. When the uORF is translated, the 40S ribosomal subunit, along with several initiation factors, can remain associated to the mRNA, resume scanning and reinitiate, at either a proximal or distal AUG codon. Translation reinitiation efficiency is dependent on the length/time taken to translate a uORF and on the length of the intercistronic region. The probability of occurring reinitiation is greater when the uORF is short or has a higher rate of translation, because some initiation factors are still associated with the 40S ribosomal subunit, allowing the recognition of a downstream AUG [9–11]. On the other hand, the length of the intercistronic region is important since the eIF2/GTP/Met-tRNA$_i$ ternary complex was used to initiate uORF translation and hence has to be reacquired *de novo.* As a result, the eIF2α is one of the modulators of the reinitiation efficiency [12–14]. In fact, the protein kinases that phosphorylate eIF2α are activated during stress conditions, resulting in global inhibition of translation [1]. However, the phosphorylation of eIF2α selectively promotes translational upregulation of a subset of mRNAs that contain uORFs, either by altering leaky scanning or, in the case of a transcript with multiples uORFs, reinitiation efficiency [15–17].

Human erythropoietin (EPO) has been the focus of many studies since it was discovered. Initially, the main function attributed to EPO was the stimulation of erythropoiesis. However, EPO has proven to be a more complex protein, having also non-hematopoietic functions, such as angiogenesis, stimulation of proliferation and anti-apoptosis [18–20]. The first site known to produce and secrete EPO was the kidney in the adult. Indeed, it is responsible for the most part of circulating EPO. *EPO* mRNA expression has also been detected in the brain (neurons and glial cells), the lung, the heart, the bone marrow, the spleen, the hair follicles, and the reproductive tract [21–26]. In these tissues, EPO has anti-inflammatory properties. For all this, EPO is known for its neuro and cardioprotective activities and has been used for the treatment of many disorders, such as cardiac and cerebral ischemia, and Alzheimer's disease [27–29].

Due to its complexity and differential expression in different organs, we can expect a tight regulation of EPO expression. Actually, EPO is known to be markedly up-regulated by hypoxia [18,30]. Both transcriptional and post-transcriptional mechanisms are able to change the EPO expression, in order to increase its levels during stress conditions. Hypoxia inducible factor 1 (HIF1) is the most well-studied factor responsible for the increase of *EPO* transcription during hypoxia [31–34].

We have previously characterized a translational mechanism controlling expression of EPO protein. We have shown that a 14-codon uORF, present in the 5' leader sequence of the *EPO* transcript, is recognized by the translational machinery, thus negatively affecting EPO expression. Also, we have observed that both leaky scanning and reinitiation are involved in the recognition of the main ORF. However, the uORF acts in peptide-independent manner and does not trigger NMD.

Another conclusion from our previous work is that *EPO* 3'UTR is able to increase the translation efficiency of the main ORF in HEK293 and in HepG2 cells, but not in HeLa cells, which suggests a different regulatory mechanism according to the tissue of EPO expression. Yet, when both uORF and 3'UTR are present in the transcript, they seem to have independent roles on EPO expression. An interesting discovery was that the uAUG of *EPO* uORF is less recognized during hypoxia, increasing EPO production via eIF2α phosphorylation. This was observed only under hypoxia in HeLa cells, which stand for a tissue- and stimuli-specific regulation of *EPO* uORF [35].

Bearing these data in mind and knowing that EPO protein is also expressed in neuronal tissue with neuroprotective functions, we were prompted to analyze whether *EPO* uORF plays a role in the regulation of EPO expression in neuronal cells. Here, we report that uORF negatively regulates expression of the main ORF in the same extent to what we have observed in other cell lines [35]. However, *EPO* mRNA levels decrease during chemical ischemia, whereas EPO protein expression is maintained, indicating that translational efficiency increases in this tissue.

MATERIALS AND METHODS

Plasmid constructs. The pGL2-Luc, pGL2-WT, pGL2-no_AUG, pGL2-no_uSTOP, pGL2-optimal_uAUG, pGL2-frameshift, pGL2-Luc-3'UTR, pGL2-WT-3'UTR and pGL2-no_uAUG-3'UTR constructs were generated by us as described previously [35].

Cell culture and plasmid transfection. SW1088 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were grown at 37°C in humidified incubator containing 5% CO_2. Transient reverse transfections were performed using Lipofectamine 2000 Transfection Reagent (Invitrogen), following the manufacturer's instructions, in 35-mm plates. Cells were co-transfected with 750 ng of the test DNA construct corresponding to the pGL2-Luc, pGL2-WT, or its derivative plasmids, and 500 ng of the pRL-TK plasmid (Promega), which encodes *Renilla* luciferase as an internal control, and, then, harvested after 24h. To mimic chemical ischemia, 20h post-transfection, the cultures were changed to fresh medium supplemented with 10 µM 2-deoxy-D-glucose (Calbiochem) and 10 µM sodium azide (Sigma).

Luminometry assay. Lysis was performed in all cell lines with Passive Lysis Buffer (Promega). The cell lysates were used to determine luciferase activity with the Dual-

Luciferase Reporter Assay System (Promega) and a Lucy 2 luminometer (Anthos Labtec), according to the manufacturer's standard protocol. One µg of extract was assayed for firefly and *Renilla* luciferase activities. Ratio is the unit of firefly luciferase after normalized with *Renilla* luciferase, and each value was derived from three independent experiments.

RNA isolation. Total RNA from transfected cells was isolated using the Nucleospin RNA extraction II kit (Marcherey-Nagel), following the manufacturer's instructions. Then, all RNA samples were treated with RNase-free DNase I (Ambion) and purified by phenol:chloroform extraction.

Reverse transcription-quantitative PCR (RT-qPCR). Synthesis of cDNA was carried out using 1µg of total RNA and Superscript II Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. Real-time PCR was performed in ABI Prism 7000 Sequence Detection System, using SybrGreen Master Mix (Applied Biosystems as previously described [35]. Quantification was performed using the relative standard curve method (ΔΔCt, Applied Biosystems). The following cycling parameters were used: 10 min at 95°C and 40 cycles of 15 sec at 95°C and 1 min at 61°C. Technical triplicates from three to four independent experiments were assessed in all cases.

Statistical analysis. Results are expressed as mean ± standard deviation. Student's *t* test was used for estimation of statistical significance. Significance for statistical analysis was defined as a $p < 0.05$.

RESULTS

EPO uORF represses translation in neuronal cells

The majority of uORFs are regulatory elements with a negative influence on translation of the main ORF [4]. Human *EPO* transcript presents a 14-codon uORF conserved among species both at the nucleotide and peptide sequence. We have previously shown that the human *EPO* uORF is functional and that it is able to decrease translation of the main ORF in about 3-fold in HEK293, HepG2 and HeLa cell lines [35] that mimic the major sites of production and secretion of EPO, the kidney in the adult and the liver in fetal life [22,36]. However, the *EPO* transcript has also been detected in other organs such as in neurons and glial cells [24,37]. This raised the question whether *EPO* uORF is also repressive in the neuronal cells. To test this hypothesis, the intact human *EPO* 5' leader sequence was cloned into the pGL2 expression vector, flanking the FLuc reporter gene to create the pGL2-WT construct (Figure 1.A). In addition, the *EPO* uORF was disrupted by site directed mutagenesis of the uAUG (ATG→TTG), using the previous pGL2-WT construct as template, originating the pGL2-no_uAUG construct (Figure 1.A). Expression of each of these reporter gene constructs was studied in a cell line derived from fibroblasts of the human brain (SW1088). For that, cellular extracts were prepared and assayed for luciferase activity and total RNA

was isolated to quantify the relative luciferase mRNA levels by RT-qPCR. The FLuc activity of each construct was normalized to the activity units of RLuc expressed from the co-transfected pRL-TK plasmid and normalized to the corresponding mRNA levels (Figure 1.B). The relative luciferase activity was compared to that of the empty pGL2-WT vector (Figure 1.A), arbitrary set as 1 (Figure 1.B). Our results show that, in SW1088 cells, human *EPO* 5' leader sequence with the intact uORF induces a 3-fold repression of translation of the reporter transcript, when compared with the relative luciferase activity from the pGL2-no_uAUG construct with no functional uORF (Figure 1.B). Thus, intact *EPO* uORF induces a repression of protein expression at the translational level, in neuronal cells.

Translation reinitiation is the mechanism by which the main ORF is mainly recognized in neuronal cells

Leaky scanning and translation reinitiation are the two described mechanisms that allow expression of the main ORF when a functional uORF is present [2]. Previously, we have observed that both uAUG leaky scanning and reinitiation, after translation of *EPO* uORF, are responsible for the translation of the main ORF in HEK293, HepG2 and HeLa cells [35]. Here, we intended to verify whether the same mechanisms act in SW1088. To evaluate these mechanisms we first mutated the stop codon of the uORF (TGA→AGA; pGL2-no_uSTOP construct), creating an extended uORF that terminates at the next in-frame stop codon, 83 nucleotides downstream from the FLuc initiation codon (pGL2-no_uSTOP construct; Figure 2.A). This mutation allows evaluating the possibility of ribosome leaky scanning since it completely abrogates the possibility of FLuc to be produced by reinitiation after translation of the uORF. In addition, we mutated in the pGL2-WT vector, the context of the uAUG codon (gggAUGa→gccAUGg), to obtain the pGL2-optimal_uAUG construct (Figure 2.A) with a uAUG sequence context shown by Kozak to yield maximum initiation frequency in higher eukaryotes [38–40]. In this case, the majority of the ribosomes load on the *EPO* mRNA 5' leader sequence are unable to leak past the uAUG codon and most likely they translate the uORF and may reinitiate at the downstream AUG codon. SW1088 cells were transiently co-transfected with pRL-TK and the pGL2-WT, pGL2-no_uSTOP or with pGL2-optimal_uAUG construct and translational efficiencies were monitored by relative luciferase activity normalized to the corresponding mRNA levels, as before. Results were compared to those obtained from the pGL2-WT construct (Figure 2.B). As shown in Figure 2.B, mutation of the uORF stop codon (pGL2-no_uSTOP construct) reduces the translation efficiency to approximately 20% of that of the pGL2-WT construct. This suggests that the percentage of ribosomes that leak past the uORF is low and thus translation of the main ORF mostly occurs by reinitiation of the ribosomes after translation termination of the uORF. In fact, analysis of the pGL2-optimal_uAUG expression allowed us to understand that translation reinitiation at the main ORF can

account for about 50% of translation efficiency, in comparison to that of the pGL2-WT construct (Figure 2.B).

In neuronal cells, the translational machinery is not blocked by the *EPO* uORF-encoded peptide

Some uORFs have the ability to induce a blockade of the translational machinery increasing their inhibitory effect. These uORFs function in a peptide-dependent manner [41,42]. The sequence of the *EPO* uORF-encoded peptide is conserved among mammalian species, indicating a putative function of this region. However, when we frameshifted the nucleotide sequence of the *EPO* uORF, in order to produce a different peptide sequence, no blockade of the translational machinery in HEK293, HepG2 and HeLa cell lines was observed [35]. In spite of that, we have investigated whether this is preserved in the cell model used in this study. For that, we used the pGL2-frameshift construct (Figure 3.A.) in which the uORF was modified by shifting the reading frame to generate a different amino acid sequence while preserving the uAUG context and most of the nucleotide sequence. The pGL2-frameshift construct was used to transiently transfect SW1088 cells. The translation efficiency obtained by each conctruct was analyzed as previously and the results were compared to those of the pGL2-WT construct (Figure 3.B.). Our data show that the mutant uORF does not derepress translation of the main ORF; instead, it decreases the translation efficiency of the main ORF (Figure 3.B.). We propose that this is due to a decrease of the reinitiation efficiency, which might be a result of the introduction of a rare codon in the altered uORF of pGL2-frameshift construct that might increase the time of translation. Thus, we can conclude that the native *EPO* uORF functions in a peptide sequence-independent manner in SW1088 cells.

In neuronal cells, *EPO* 3'UTR has no impact on the inhibitory effect of the uORF

It is known that mRNA circularization brings in close proximity the 5' leader sequence and the 3'UTR of a transcript and increases translation efficiency. Here we aimed to prove the effect of the *EPO* 3'UTR in SW1088 cells. The pGL2-Luc-3'UTR construct (Figure 4.A) was transiently transfected into SW1088 cells. Translation efficiency was obtained has before and the pGL2-Luc-3'UTR was compared to that of the empty pGL2-Luc construct. The results show that the *EPO* 3'UTR alone induces about a 5-fold increase of the translation efficiency (Figure 4.B). Thus, the *EPO* 3'UTR-containing construct in SW1088 cell line has the same effect observed for HEK293 and HepG2 cell lines, highlighting the differential regulation of these structures in the HeLa cell line. In addition, our results show that the *EPO* uORF retains its repressive impact on the main ORF translation even in the presence of 3'UTR (Figure 4C. and D). Indeed, the intact *EPO* 5' leader sequence in the pGL2-WT-3'UTR construct allows a significant 3-fold decrease in translation efficiency when compared to that observed from the pGL2-no_uAUG-3'UTR construct with the disrupted uORF (Figure 4.D). Thus, the *EPO* 3'UTR fails to overcome translational repression induced by the *EPO* uORF in SW1088 cells.

The repressive effect of the *EPO* uORF is inhibited during chemical ischemia

Stress conditions can lead to dramatic changes on the overall protein synthesis. In general, there is a global decrease on protein synthesis, but there is growing evidence that some mRNAs can be specifically controlled in order to alter their expression patterns. Selective subsets of mRNAs that are shown to overcome this global pressure present in their sequence regulatory elements such as uORFs or IRESes [46–48]. In order to understand the neuronal relevance of the *EPO* uORF, we have decided to assess whether this structure alters the main ORF expression in response to ischemic conditions. For that, SW1088 cells were transiently transfected with the pGL2-WT and pGL2-no_uAUG constructs that carry the intact or disrupted *EPO* uORF (Figure 5.A), respectively; then, cells were treated with 10µM of 2-deoxy-D-glucose and 10 µM of sodium azide, to induce chemical ischemia. Six hours later, cells were lysed and protein and RNA were extracted and analysed by luciferase assays and RT-qPCR, as previously. Our results show that the translation efficiency of pGL2-WT construct has a 4-fold increase under chemical ischemia (Figure 5.B).

DISCUSSION

EPO regulates the proliferation, differentiation and death of the erythroid cells [49]. Due to its ability to promote cell survival and differentiation it acts in non-hematopoietic cells modulating proliferation and cellular viability [20,50]. Although, the major site of EPO production is the kidney in the adult, many organs have been described to express the *EPO* mRNA [21,23,26,51]. In those organs, EPO seems to act locally not contributing to erythropoiesis. Since EPO is expressed in cardiac and neuronal cells it has been described as cardio and neuroprotective. Moreover, EPO is regulated at multiple levels to ensure its correct response to external stimuli in different tissues.

EPO gene expression is best studied at transcriptional level. Promoter silencing in the adult liver leads to a change of the site of production from the liver to the kidney [52]. Many other mechanisms control EPO expression, such transcriptional activation of *EPO* gene by HIF1 during hypoxic conditions [31,53–55]. The higher levels of circulating EPO, as a result of this stimulation, increases the red blood cell mass in order to rise the oxygen-carrying capacity of the blood [50]. Although, this hypoxic activation has been described mainly in the kidney and liver, there is also an oxygen-dependent regulation of EPO expression in the brain [37]. This fact, together with the neuronal expression of EPO and EPO receptor (EPOR) and the protection effects of this signalling pathway during stroke, brain injury or cerebral ischemia, suggests a paracrine function of EPO in the neuronal tissue and a regulation of EPO expression in these cells [50,56,57].

Previously we have shown that the *EPO* transcript presents in its 5' leader sequence a highly conserved 14-codon uORF that acts as a negative regulatory element able to decrease the expression of the main ORF in about 3-fold in HEK293, HepG2 and HeLa cell lines [35]. Since, the EPO protein is expressed in the neuronal tissue and seems to have specific neuroprotective functions, we aimed to study *EPO* uORF-mediated regulation in these cells lines. We found that the *EPO* uORF is functional also in neuronal cell lines decreasing the main ORF expression in about 3-fold (Figure 1).

Also, we observed that reinitiation and, in a less extent, leaky scanning are both mechanisms implicated in the recognition of the *EPO* AUG in neuronal cells (Figure 2). We have also shown that the repressive effect exerted by the *EPO* uORF does not depend on the encoded peptide (Figure 3). In adition, the *EPO* 3'UTR does not affect the repressive ability of the uORF (Figure 4). Under chemical ischemia, the *EPO* uORF repression is completely abrogated (Figure 5). This suggests the existence of a different mechanism for translational regulation from the one observed in HeLa cells [35].

Overall, we have explained the regulatory mechanism of the human *EPO* uORF in neuronal tissue. The fact that the basic mechanism is preserved reveals that this structure thoroughly regulated the human *EPO* expression, although its response to chemical ischemia shows a different regulatory mechanism.

Further studies might bring new insights on the modulation of human *EPO* expression, particularly in the brain, encouraging the development of new forms of therapy for many neurodegenerative diseases.

ACKNOWLEDGEMENTS

We are grateful to Margarida Gama Carvalho, for supplying the pRL-TK plasmid, and to the Oncology group at Instituto Nacional de Saúde Dr. Ricardo Jorge for providing the SW1088 cell line. We would also like to thank Isabel Peixeiro, Cláudia Onofre, João Lavinha e Rafaela Lacerda for critical reading of the manuscript. This research was partially supported by Fundação para a Ciência e a Tecnologia (PEst-OE/BIA/UI4046/2011, PTDC/BIM-MED/0352/2012 and SFRH/BD/63581/2009 to C.B.).

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FIGURE LEGENDS

Figure 1. The *EPO* uORF represses translation of the downstream main ORF in neuronal cells.

(A) Schematic representation of reporter constructs as in figure II.1. The human *EPO* 5' leader sequence encompassing its uORF (open box) with the intact initiation (uAUG) and termination (UGA) codons, was cloned upstream of the firefly luciferase coding region (FLuc; grey boxes) to create the pGL2-WT construct. In the pGL2-no_uAUG construct, the uORF initiation codon is mutated (AUG→UUG) (the cross represents the point mutation and the dashed lined box represents the non-functional uORF). **(B)** The *EPO* 5' leader sequence represses protein expression of the downstream reporter. SW1088 cells were transiently co-transfected with each one of the constructs described in (A) and with the pRL-TK plasmid encoding the *Renilla* luciferase (RLuc). Cells were lysed twenty-four hours later and the luciferase activity was measured by luminometry assays. FLuc activity values were normalized to RLuc activity to control

for transfection efficiency. In parallel, the luciferase mRNA levels were quantified by RT-qPCR. The graph represents the data as translational efficiency (relative luciferase activity/mRNA levels). Expression levels obtained from pGL2-WT construct were defined as one. Average values and standard deviation (SD) of three independent experiments are shown. Statistical analysis was performed using Student's *t* test (unpaired, two tailed); (∗) $p<0.05$; (∗∗) $p<0.01$; (∗∗∗) $p<0.001$.

Figure 2. Mainly, the downstream main AUG codon is recognized by translation reinitiation.

(A) Schematic representation of reporter constructs, as in figure II.2. The pGL2-WT plasmid contains the wild-type human *EPO* 5' leader sequence, the pGL2-no_uSTOP construct presents the *EPO* 5' leader sequence with a mutation (UGA→AGA) at the uORF translation termination codon, which makes the uORF to overlap with the luciferase ORF (the cross represent the point mutation), and the pGL2-optimal_uAUG contains the *EPO* 5' leader sequence with a optimal uAUG sequence context (gggAUGa→gccAUGg; represented by a bold lined box). **(B)** SW1088 cells were transiently co-transfected with each one of the constructs described in (A) and with a plasmid encoding *Renilla* luciferase (pRL-TK) and analyzed as described in the legend to Figure 1.

Figure 3. In neuronal cells, the translational repression exerted by the *EPO* uORF is peptide sequence-independent.

(A) Schematic representation of the expression constructs. The pGL2-WT plasmid contains the human normal *EPO* 5' leader transcript sequence, the pGL2-frameshift vector carries a EPO uORF sequence modified by frameshift mutations, which consist in the insertion of one nucleotide in the second codon (+1 nt) and the deletion of one nucleotide in 13^{th} codon (-1 nt). The resulting uORF-encoded peptide sequence is shown below. **(B)** SW1088 cells were transiently co-transfected with each one of the constructs described in (A) and with a plasmid encoding *Renilla* luciferase (pRL-TK) and analyzed as described in the legend to Figure III.1.B.

Figure 4. In neuronal cells, the 3'UTR of the *EPO* mRNA has no influence in the inhibitory effect of the uORF.

(A) Schematic of the firefly luciferase (FLuc) reporter constructs containing the native luciferase 3'UTR (pGL2-Luc) or the 3'UTR sequence (dark grey box) of the human *EPO* transcript (pGL2-Luc-3'UTR). **(B)** SW1088 cells were transiently co-transfected with each one of the constructs described in (A) and with a plasmid encoding *Renilla* luciferase (pRL-TK) and analyzed as described in the legend to Figure 1. **(C)** Schematic of the firefly luciferase (FLuc) reporter constructs containing the human *EPO* 5' leader

sequence with the intact uORF and the 3'UTR sequence (dark grey box) of the human *EPO* transcript (pGL2-WT-3'UTR), or the *EPO* 5' leader sequence with a disrupted uORF due to the uAUG→UUG mutation (represented by a cross) and the EPO 3'UTR sequence (dark grey box; pGL2-no_uAUG-3'UTR). **(D)** SW1088 cells were transiently co-transfected with each one of the constructs described in (C) and with a plasmid encoding *Renilla* luciferase (pRL-TK) and analyzed as described in the legend to Figure 1.

Figure 5. In neuronal cells under chemical ischemia, EPO uORF-mediated translational repression is released.

(A) Schematic representation of the pGL-WT and pGL2-no_uAUG vectors represented as in Figure 1. These constructs were separately co-transfected with a plasmid encoding *Renilla* luciferase (pRL-TK) in SW1088 cells. Twenty hours later cells were untreated (-) or treated (+) with 10 µM 2-deoxy-D-glucose and 10 µM sodium azide. **(B)** Untreated (CI: -) and treated (CI: +) transfected cells were lysed and analyzed as described in the legend to Figure 1. The dark bars correspond to the pGL2-no_uAUG construct and the light bars to the pGL2-WT.

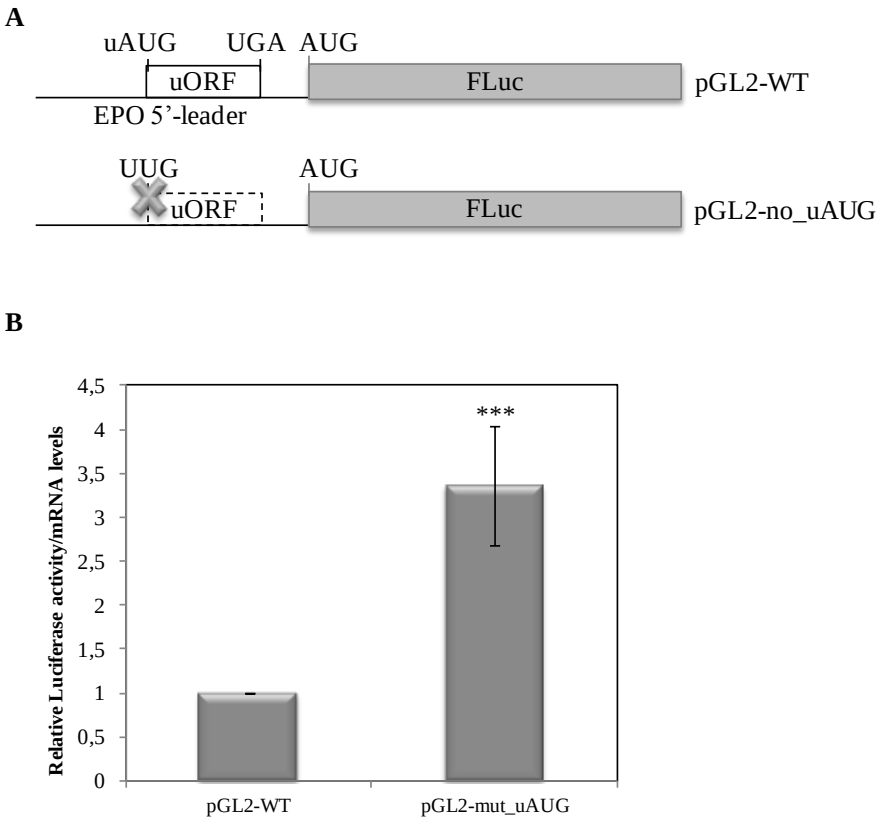
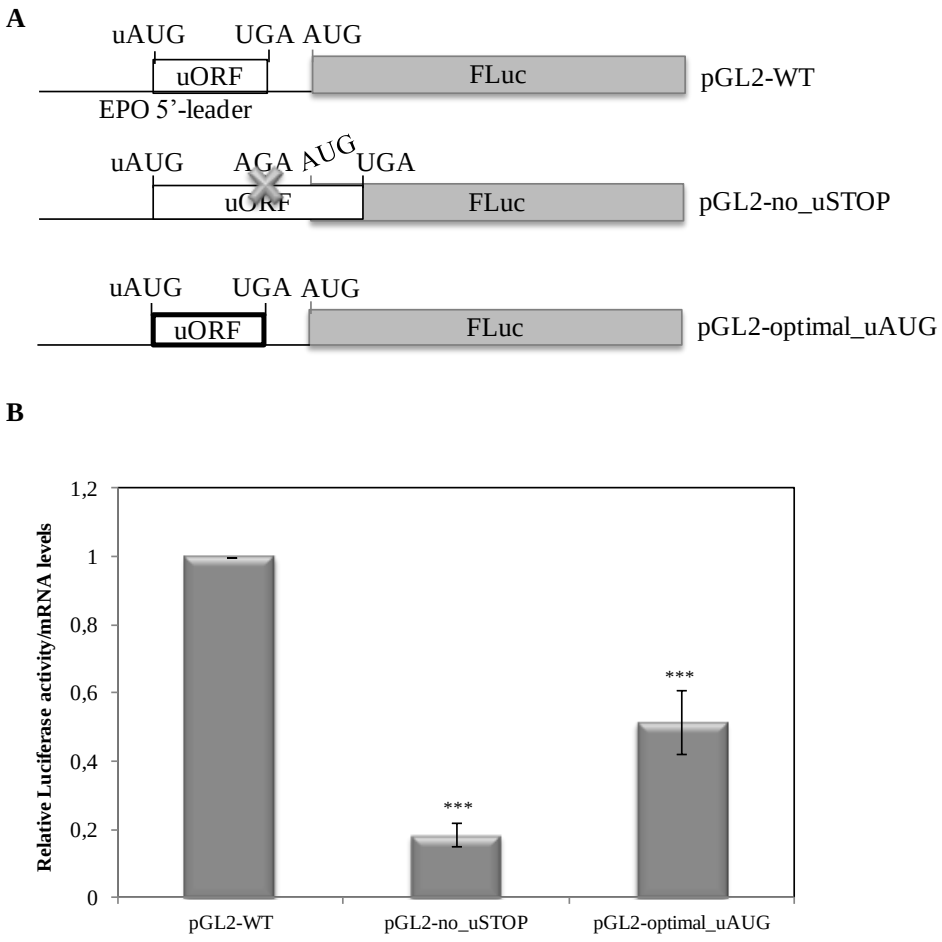


Figure 1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EPO 5'-leader
uAUG
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UGA
uORF
FLuc
pGL2-no_uSTOP
uAUG
UGA AUG
uORF
FLuc
pGL2-optimal_uAUG
B
Relative Luciferase activity/mRNA levels
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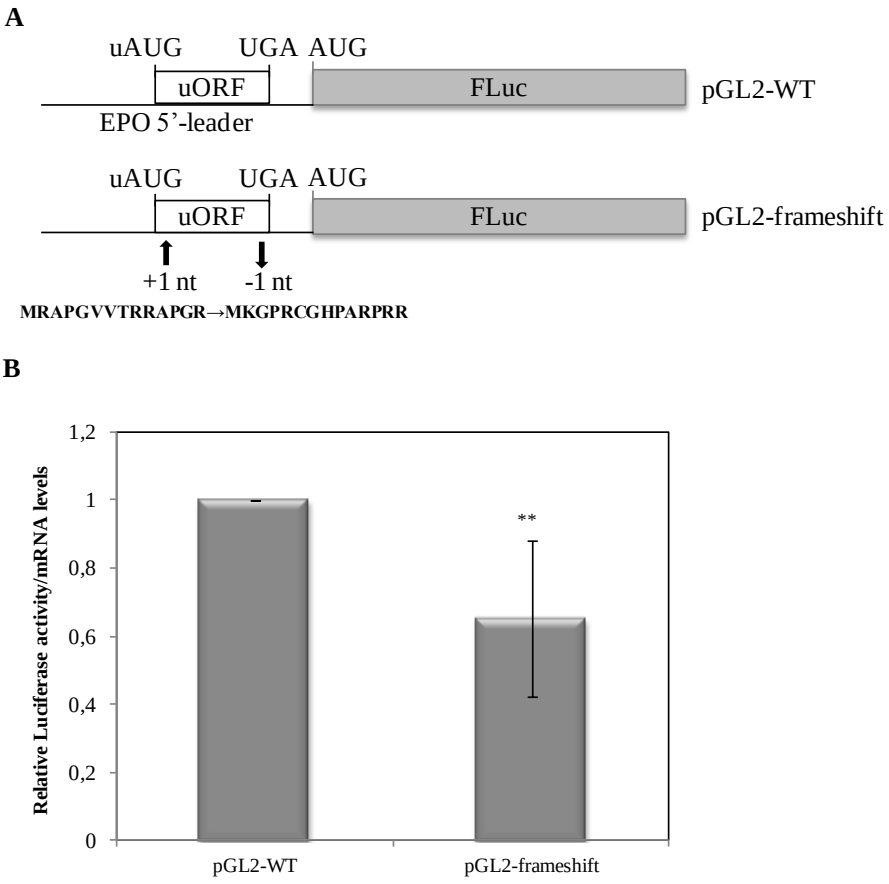
pGL2-WT
pGL2-no_uSTOP
pGL2-optimal_uAUG

Figure 2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UGA
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EPO 5'-leader
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UGA
AUG
uORF
FLuc
pGL2-frameshift
+1 nt
-1 nt
MRAPGVVTRRAPGR→MKGPRCGHPARPRR
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Relative Luciferase activity/mRNA levels
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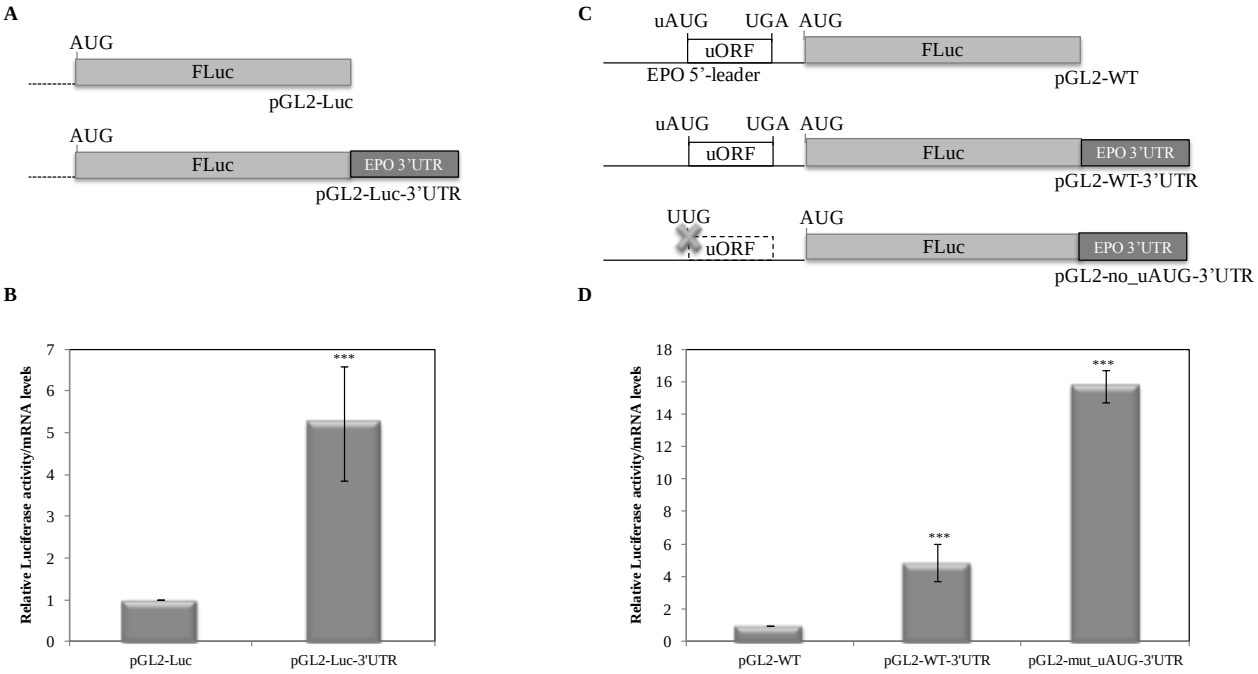
pGL2-WT
pGL2-frameshift

Figure 3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uORF
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EPO 5'-leader
pGL2-WT
uAUG
UGA
AUG
uORF
FLuc
EPO 3'UTR
pGL2-WT-3'UTR
UUG
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pGL2-no_uAUG-3'UTR
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Relative Luciferase activity/mRNA levels
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pGL2-Luc
pGL2-Luc-3'UTR
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Relative Luciferase activity/mRNA levels
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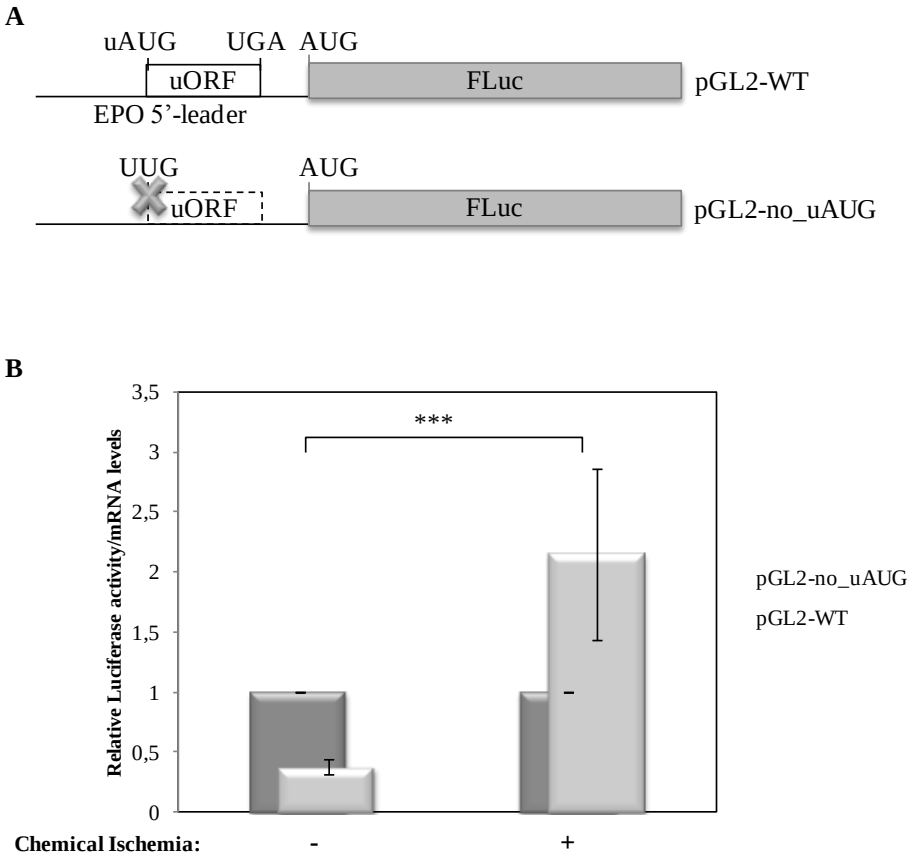
pGL2-WT
pGL2-WT-3'UTR
pGL2-mut_uAUG-3'UTR

Figure 4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B
Relative Luciferase activity/mRNA levels
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2
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pGL2-no_uAUG
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Chemical Ischemia:
-
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Figure 5

Translation of the human erythropoietin transcript is regulated by an upstream open reading frame in response to stress in cardiac and muscle cells

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Key words: erythropoietin (EPO); hypoxia; ischemia; translational regulation; upstream open reading frame (uORF); heart; muscle

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INTRODUCTION

The regulation of genetic expression comprises several key processes that maintain and adapt to the cell needs. One of these key processes is without a doubt the translation. This mechanism has the ability to rapidly overcome stress through modification of its basic translation machinery or by maintaining certain mRNAs over others. Several features of the 5' untranslated region (5'UTR) of the mRNA are important for its translational regulation such as upstream open reading frames (uORFs) and internal ribosome entry sites (IRESes) (Sonenberg & Hinnebusch 2009; Pichon et al. 2012; Somers et al. 2013). uORFs widely spread on the genome are *cis*-acting regulatory elements (Calvo et al. 2009). Their role on the translational regulation has been shown in several studies that demonstrate how the introduction or deletion of a uORF can cause disease (Wethmar et al. 2010; Barbosa et al. 2013).

In order to be functional a uORF has to be recognized by the translational machinery. In this matter, the context of the upstream AUG (uAUG) must be GCC(A/G)CCAUGG, where the -3 and +4 are the most important positions (Kozak 1987). When the uAUG context lacks a suitable nucleotide in either -3 and/or +4 position, the uORF recognition is regulated by a so-called leaky scanning mechanism where the ribosomes can either scan through the uAUG codon or recognize it, initiating translation (Kozak 1991). Once the uORF is recognized and translated the nascent peptide can stall through direct interaction between the translating ribosome and the uORF-encoded peptide during either the elongation or termination phase, creating a blockade to additional scanning ribosomes (Sachs & Geballe 2006; Meijer & Thomas 2002). Finally, when the ribosome reaches the stop codon two fates may arise, the translation terminates and the ribosome leaves the mRNA or the ribosome can resume scanning and reinitiate in a AUG further downstream (Morris & Geballe 2000; Sachs & Geballe 2006).

In its classical hormone role, human erythropoietin (EPO) is a glycoprotein synthesized and released mainly from kidney, which controls erythropoiesis (Jelkmann 1992). However, several studies have revealed that many tissues (e.g. brain, heart and liver) have the ability to produce EPO, granting an advantage to rapidly respond to different cell stress stimuli and tissue injuries. For example, EPO is upregulated in the brain and heart after injury, promoting a tissue protection due to its antiapoptotic, anti-inflammatory, antioxidative and angiogenic properties (Ruifrok et al. 2010; Brines & Cerami 2008; Mowat et al. 2012; Arcasoy 2008; Ryou et al. 2012). Thus, it is clear that EPO has a therapeutic potential in the treatment of several human disorders, including ischemic injuries in brain or heart (Ruifrok et al. 2010; Brines & Cerami 2008; Mowat et al. 2012; Arcasoy 2008; Ryou et al. 2012).

It is well known that the EPO expression is tightly controlled, being markedly up-regulated by hypoxic conditions that increase the EPO serum levels up to several fold (Besarab et al. 2009; Chin et al. 2000). Hypoxia inducible factor 1 (HIF1) is the responsible for augmenting the EPO transcriptional rate due to its binding to the hypoxia-responsive element located in the 3' untranslated region (UTR) of the *EPO* gene (Noguchi et al. 2011; Wang et al. 1995). During hypoxia, besides the HIF1 production it is also activated a family of protein kinases that phosphorylate eIF2α. This eIF2α phosphorylation has a great impact on the protein synthesis and is also observed in other stress situations such as pathogenic infection, chemical exposure, and nutrient starvation (Sonenberg & Hinnebusch 2009). In these situations, despite of a decrease in general protein synthesis there are some transcripts that are selectively upregulated, such as transcripts bearing uORFs (Onofre et al. 2015; Palam et al. 2011; Pöyry et al. 2004; Somers et al. 2013; Barbosa & Romão 2014).

EPO is a 156 amino acid protein encoded by 1340 nucleotides transcript (NM_000799) that presents a 5'UTR with 181 nts containing a uORF with 14 codons that terminates 22 nts upstream of the EPO AUG codon. Here we report that this single uORF located in the human EPO mRNA inhibits translation in unstressed muscle and heart cells. However, this repression is significantly released by hypoxia and ischemia in muscle cells where in the heart cells this release is only observed in hypoxic situations.

MATERIALS AND METHODS

Plasmid constructs. Most plasmids used in this work were previously described (Barbosa & Romão, 2014). The WT-FLAG construct carries a single FLAG-tag sequence immediately before the stop codon. This construct was obtained by site-directed mutagenesis.

Cell culture and plasmid transfection. C2C12 and H9c2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco). Cells were grown at 37^{o}C in humidified incubator containing 5% CO2. Transient transfections were performed using Lipofectamine 2000 Transfection Reagent (Invitrogen), following the manufacturer's instructions, in 35-mm plates. Cells were cotransfected with 500 ng of the test DNA construct corresponding to the pGL2-Luc, pGL2-WT, or its derivative plasmids, and 500 ng of the pRL-TK plasmid (Promega), which encodes Renilla luciferase as an internal control, and, then, harvested after 24h. To mimic ischemia, 20h and 16h post-transfection, the C2C12 and H9c2 cultures were changed to fresh medium supplemented respectively with 3 mM and 8µM Sodium iodoacetate (Sigma-Aldrich). To activate eIF2α kinases and induce eIF2α phosphorylation, 4h after transfection, cells were treated during 20h with 1µM thapsigargin (Enzo-Life Technologies). To mimic hypoxia the C2C12 and H9c2 cells were changed to fresh medium supplemented with 200µM of cobalt chloride ($CoCl_2$) 15h

and 6h post-transfection respectively until the 24h of transfection were achieved (Sigma-Aldrich).

SDS-PAGE and Western blotting. Protein lysates were resolved, according to standard protocols, in 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad). Membranes were probed using mouse monoclonal anti-α-tubulin (Sigma) at 1:10000 dilution, rabbit polyclonal anti-eIF2α (Cell Signaling) at 1:500 dilution, rabbit polyclonal anti-phospho Ser 51 eIF2α (Life Technologies) at 1:500 dilution or rabbit polyclonal anti-HIF1α (BD Biosciences) at 1:500. Detection was carried out using secondary peroxidase-conjugated anti-mouse IgG (Bio-Rad), anti-rabbit IgG (Bio-Rad) antibodies followed by chemioluminescence.

Luminometry assay. Lysis was performed in all cell lines with Passive Lysis Buffer (Promega). The cell lysates were used to determine luciferase activity with the Dual-Luciferase Reporter Assay System (Promega) and a Glomax luminometer, according to the manufacturer's standard protocol. One μg of extract was assayed for firefly and *Renilla* luciferase activities. Ratio is the unit of firefly luciferase after normalized with Renilla luciferase, and each value was derived from three independent experiments.

RNA isolation. Total RNA from transfected cells was isolated using the Nucleospin RNA extraction II kit (Marcherey-Nagel), following the manufacturer's instructions. Then, all RNA samples were treated with RNase-free DNase I (Promega) and purified by phenol:chloroform extraction.

Reverse transcription-quantitative PCR (RT-qPCR). Synthesis of cDNA was carried out using 1μg of total RNA, NZY Ribonuclease Inhibitor (NZYTech) and NZY Reverse Transcriptase (NZYTech), according to the manufacturer's instructions. Real-time PCR was performed in ABI Prism 7000 Sequence Detection System, using SybrGreen Master Mix (Applied Biosystems). Primers specific for the firefly luciferase cDNA (primers #11 and #12; Table 1) and *Renilla* luciferase cDNA (primers #13 and #14; Table 1), were designed by Barbosa and Romão 2014. Quantification was performed using the relative standard curve method (ΔΔCt, Applied Biosystems). The luciferase mRNA levels were normalized to *Renilla* luciferase mRNA levels for transfection efficiency. The following cycling parameters were used: 10 min at 95°C and 40 cycles of 15 sec at 95°C and 1 min at 61°C. Technical triplicates from three to four independent experiments were assessed in all cases.

Transgenic mice. The DNA fragments for pronuclear microinjection in C57B1/6 mice were obtained by digestion of the pGL2-WT and pGL2-no_uAUG constructs with KpnI (Thermo Scientific) and SalI (Thermo Scientific) restriction enzymes resulting a fragment with 3799 kb containing the CMV promoter, the human EPO 5'UTR, the Luciferase cistron and the poly-A tail. These fragments were purified by the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions.

Statistical analysis. Results are expressed as mean ± standard deviation. Student's *t* test was used for estimation of statistical significance. Significance for statistical analysis was defined as a $p<0.05$.

RESULTS

The EPO uORF represses translation in muscle and heart cells

A previous work in our lab showed that the 5'UTR sequence of the EPO has a conserved uORF with 14 codons that represses translation at a downstream AUG (Barbosa & Romão 2014). Here we aimed to understand if the EPO uORF has the same ability in muscle and heart cells, namely in C2C12 and H9c2 cells. For that, these cell lines were transfected with the pGL2-WT construct, which contains the EPO 5'UTR, and the pGL2-no_uAUG construct where the uAUG was eliminated (ATG→TTG) (Figure 1A). The correspondent cell extracts were assayed for Luciferase activity, and total RNA was isolated to quantify the relative Luciferase mRNA levels by reverse transcription-quantitative PCR (RT-qPCR). Firefly Luciferase (FLuc) activity of each construct was normalized to the activity units from *Renilla* Luciferase (RLuc) expressed from the cotransfected pRL-TK plasmid. The relative Luciferase activity was then normalized to the corresponding mRNA levels and compared to the levels obtained with the empty pGL2-Luc vector, arbitrarily defined as one (Figure 1B). The results showed that in both cell lines the construct bearing the intact EPO 5'UTR sequence represses the translation: the EPO uORF induces a 2 fold repression in H9c2 cells and a 6 fold in C2C12 cells (Figure 1).

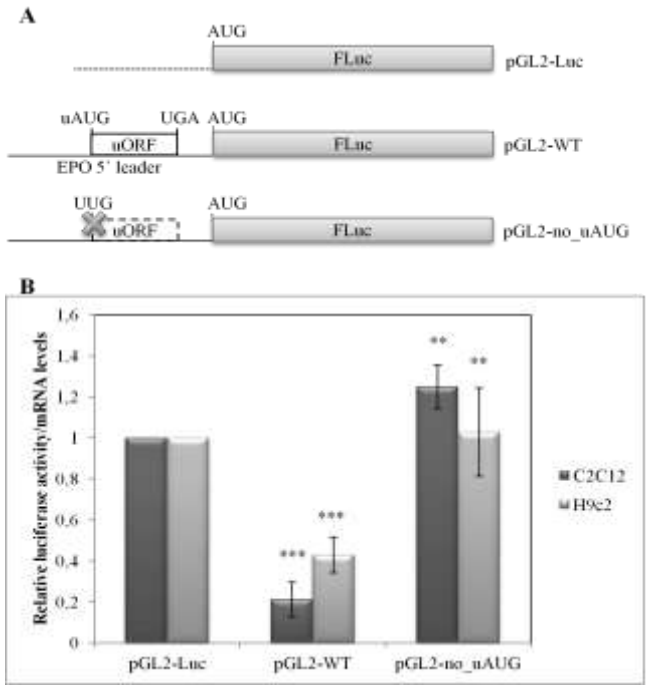

Figure 1. The EPO uORF exerts translational repression in muscle and heart cells. (A) Schematic representation of reporter constructs. The human EPO 5'-leader sequence encompassing its uORF (open

boxes) with the intact initiation (uAUG) and termination (UGA) codons, was cloned into the empty vector (pGL2-Luc), upstream of the firefly luciferase coding region (FLuc; grey boxes) to create the pGL2-WT construct. In the pGL2-no_uAUG construct, the uORF initiation codon is mutated (AUG→UUG) (the cross represent the point mutation and the dashed lined box represent the non-functional uORF). **(B)** The EPO 5'UTR sequence comprises a uORF that represses the protein expression of the downstream reporter. C2C12 and H9c2 cell lines were transiently co-transfected with each one of the constructs described in (A) and with the pRL-TK plasmid encoding the *Renilla* luciferase (RLuc). Cells were lysed twenty-four hours later and the luciferase activity was measured by luminometry assays. The luciferase mRNA levels were quantified by RT-qPCR. The relative luciferase activity of each construct was normalized to the mRNA levels. Relative Luciferase/mRNA ratio of the pGL2-Luc was defined as one. Average values and standard deviation (SD) of three independent experiments are shown. Statistical analysis was performed using Student's *t* test (unpaired, two tailed); (∗) $p<0.05$; (∗∗) $p<0.01$; (∗∗∗) $p<0.001$.

After the EPO uORF translation the main AUG is recognized mainly after translation reinitiation

Despite of the good uAUG context of the uORF (GGGAUGA), it lacks the suitable G in the position +4. It would be expected that the majority of the ribosomes scanning the 5'UTR would recognize and translate the uORF. However, some of them would leaky scan the uAUG initiating at the main AUG. For those ribosomes that translate the uORF, they can still translate the main ORF through reinitiation. To evaluate the leaky scanning and reinitiation extent, two constructs were tested. The first one, the pGL2-optimal_uAUG construct, has the uAUG in an optimal context (GGGAUGA → GCCCAUGG) to address the translation reinitiation rate (Figure 2A). The second one, the pGL2-no_uSTOP construct has the uORF stop codon mutated to a sense codon (TGA→AGA) creating a new uORF stop codon 83nt downstream from the Fluc initiation (Figure 2A). This last construct will show the leaky scanning rate since the ribosome will not be able to reinitiate because both reading frames overlap. The pGL2-optimal_uAUG, pGL2-no_uSTOP and pGL2-WT constructs were transfected in the C2C12 and H9c2 cells lines, and the translational efficiencies were monitored by dual luciferase assays normalized to the corresponding mRNA levels, as before. Results were compared to those obtained from the pGL2-WT construct (Figure 2B). Our data show that when we optimize the uAUG context the translational efficiency decreases to 80% in C2C12 and 60% in H9C2 cells. This demonstrates that most of the times that the ribosome reaches the main AUG it occurs by translation reinitiation. On the other hand, the pGL2-no_uSTOP expression decreased to 30% in C2C12 cells and 15% in H9c2 cells, allowing us to observe that a few percentage of ribosomes leak past the uORF. The Figure 2B demonstrates that a vast majority of the ribosomes are able to reinitiate translation after the uORF translation and that few ribosomes are able to perform the leaky scanning of the uAUG.

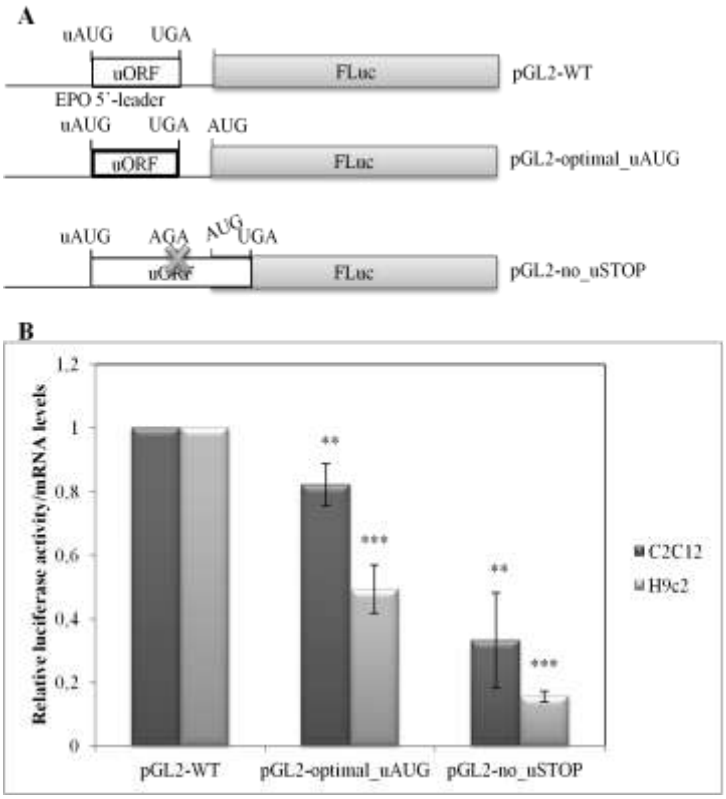

Figure 2. After the EPO uORF translation the main AUG is recognized mainly by translation reinitiation. (A) Schematic representation of reporter constructs. The pGL2-WT construct contains the native EPO 5'UTR sequence. The pGL2-no_uSTOP construct contains the EPO uORF with the uSTOP codon mutated to a sense codon (UGA → AGA), represented by the cross, originating a new uSTOP codon further downstream on the Luciferase cistron. The pGL2-optimal_uAUG construct presents the EPO uORF, represented by a bold lined box, with the uAUG in the optimal Kozak context (GGGAUGA → GCCCAUGG). **(B)** C2C12 and H9c2 cells were transiently cotransfected with the plasmids represented in A and with the pRL-TK plasmid encoding RLuc. The results were analyzed as described in the legend for Figure 1B.

The EPO uORF encoded peptide does not influence the translation repression

Since the peptide encoded by the EPO uORF is conserved (Barbosa & Romão 2014) it could indicate a functional role in the translational regulation as previous studies reported (Karagyozov et al. 2008; Onofre et al. 2015; Wei et al. 2012). To test this hipothesis in heart and muscle cells, the frame of the EPO uORF peptide was shifted maintaining most of the nucleotide sequence creating the pGL2-frameshift construct (Figure 3A), and C2C12 and H9c2 cells were transiently transfected with these constructs. The translational efficiencies were monitored and normalized as before. Results were compared to those obtained from the pGL2-WT construct (Figure 3B). The results did not showed a significant difference in translation efficiency (Figure 3B). Thus, the EPO uORF encoded peptide does not influence the translational repression exerted by the uORF.

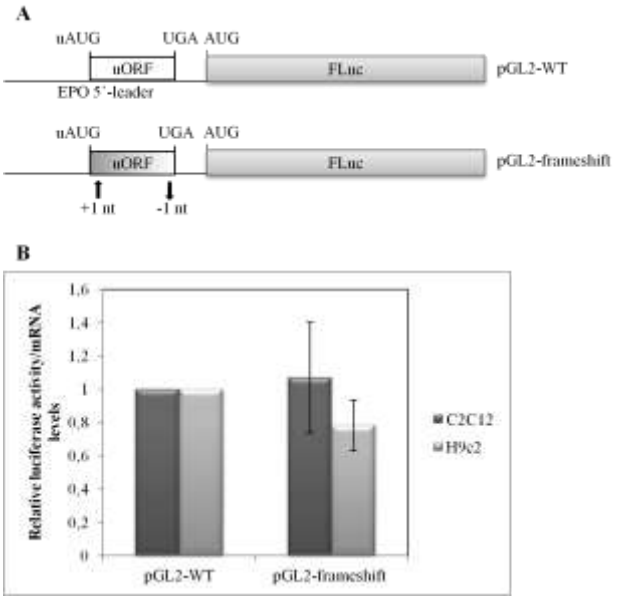

Figure 3. The EPO uORF sequence peptide does not influence the translation repression. (A) Schematic representation of reporter constructs. The pGL2-WT construct contains the native EPO 5'UTR sequence. The pGL2-frameshift construct contains the EPO uORF with a shifted reading frame altering the encoded peptide. **(B)** C2C12 and H9c2 cells were transiently cotransfected with the plasmids represented in A and with the pRL-TK plasmid encoding RLuc. The results were analyzed as described in the legend of the Figure 1B.

The EPO 3'UTR does not influence the relative EPO uORF translation repression

The EPO 3'UTR have been described as a regulator of the EPO expression by stabilizing the mRNA (Wang et al. 1995; McGary et al. 1997; Czyzyk-Krzeska & Bendixen 1999; Ohigashi et al. 1999). Additionally, it has been shown that through protein interaction between the 5' and 3' UTRs the uORF translational inhibition might be influenced (Mehta et al. 2006). In order to understand if the EPO 3'UTR can modulate the translational repression exerted by the EPO uORF we cloned the EPO 3'UTR downstream from the FLuc as shown in figure 4A and 4C. First, it was tested if the EPO 3'UTR has some effect on the translational efficiency of the FLuc by cloning the EPO 3'UTR on the pGL2-Luc construct (pGL2-Luc-3'UTR construct) (Figure 4A). This construct, and pGL2-Luc construct, were transiently transfected in the same panel of cells. The translational efficiencies were monitored and normalized as before (Figure 4B). The figure 4B shows a 2-fold increase on the translation efficiency on the C2C12 cells due to the EPO 3'UTR. On the other hand, the same 3'UTR does not influence the translation efficiency in the H9c2 cells. Then, to verify if this effect on the translation efficiency is susceptible to be influenced by the EPO 5'UTR, the EPO 3'UTR was cloned in the constructs with and without the uAUG (Figure 4C). These constructs, pGL2-WT-3'UTR and pGL2-no_uAUG-3'UTR, were transfected in parallel with the pGL2-WT construct to which the results were compared. The results show that in C2C12 cells, but not in H9c2 cells, the 3'UTR increases translation (Figure 4D). However, the uORF

maintains its ability to inhibit the translation in the presence of the 3'UTR in both cell lines.

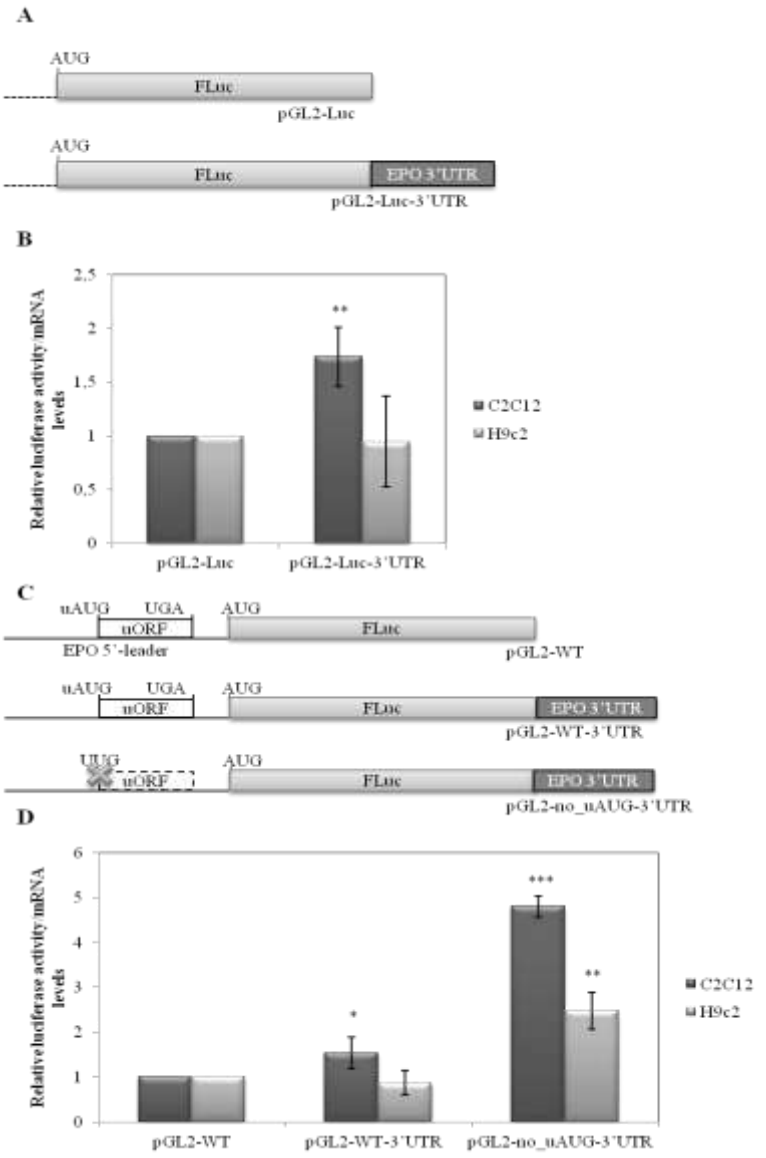

Figure 4. The EPO 3'UTR does not influence the relative EPO uORF translation repression (A). Schematic representation of the pGL2-Luc construct that contains the native luciferase 3'UTR and the pGL2-Luc-3'UTR construct where the EPO 3'UTR (dark gray box) was cloned. **(B)** C2C12 and H9c2 cells were transiently cotransfected with the plasmids represented in A and with the pRL-TK plasmid encoding RLuc. The results were analyzed as described in the legend of the Figure 1B. **(C)** Schematic representation of the FLuc reporter constructs containing the human EPO 5'UTR with either the luciferase 3'UTR (pGL2-WT) or the EPO 3'UTR (pGL2-WT-3'UTR). The pGL2-no_uAUG-3'UTR presents the EPO 5'UTR with a disrupted uORF due to the uAUG to UUG mutation (represented by a cross) and the EPO 3'UTR sequence. **(D)** C2C12 and H9c2 cells were transiently cotransfected with the plasmids represented in A and with the pRL-TK plasmid encoding RLuc. The results were analyzed as described in the legend of the Figure 1B.

The HIF expression during chemical ischemia or hypoxia seems to alleviate the translational regulation exerted by the EPO uORF

To cope with cellular stress, global protein synthesis is inhibited, but a subset of mRNAs, such as those carrying uORFs, are specifically translated (Barbosa & Romão 2014). Here we decided to investigate if the muscle and heart cells can direct the translational control in response to ischemia and hypoxia and allow human EPO uORf-mediated translational derepression, as it is wildly known EPO responds to those stresses (Haase 2013; Ebert & Bunn 1999; Li et al. 2008). Thus, our panel of cells was transfected with the pGL2-WT and pGL2-no_uAUG constructs in presence or absence of sodium iodoacetate treatment that mimics ischemia (Figure 5A). The translational efficiency was measured as before and the results were normalized to those of the pGL2-no_uAUG construct. Additionally, the ischemic stimulus was monitored by Western blot against the HIF1α and eIF2α phosphorylation, two indicators of stress (Figure 5B). The analysis by Western blot revealed that in C2C12 cells the eIF2α is phosphorylated and the HIF1α is produced under this ischemic stress. However, in H9c2 cells the sodium iodoacetate does not lead to the HIF1α production despite the increase of the eIF2α phosphorylation. The figure 5C shows an increase in the pGL2-WT construct expression under ischemia, in the muscle cells. On the contrary, the translational efficiency on the heart cells did not suffer any alteration. Additionally, we used the same stimulus to investigate if the EPO 3'UTR managed the translational regulation under this stress. The constructs pGL2-WT, pGL2-WT-3'UTR and pGL2-no_uAUG-3'UTR were transfected following the conditions above mentioned and the results were normalized those of the pGL2-WT construct (supplemental figure 1A). The efficiency of this stimulus was verified by Western blot as mentioned above (supplemental figure 1B). The translational efficiency of the constructs bearing the EPO 3'UTR did not revealed a significant change in response to ischemia in both cell lines (supplemental figure 1C).

Next, we investigated if the hypoxia is able to modulate the translation of the EPO uORF. For this purpose we transfected the same panel of cells with the pGL2-WT and pGL2-no_uAUG constructs. Then, the cells were untreated or treated with 200 μM of cobalt chloride ($CoCl_2$) that mimics hypoxia. The results were analyzed and normalized as before. The figure 5D represents the Western blot of this assay showing that HIF1α is produced in both cell lines when treated with $CoCl_2$. Additionally, it is observed that the eIF2α is phosphorylated in the presence of $CoCl_2$. The figure 5E shows an increase in the pGL2-WT construct expression under hypoxia in both cell lines.

We also investigated if hypoxia had some effect on the translational regulation in the presence of the EPO 3'UTR. The experimental procedures and result analysis were the same as before using the pGL2-WT, pGL2-WT-3'UTR and pGL2-no_uAUG-3'UTR constructs. Cells were untreated or treated with $CoCl_2$ to mimic the hypoxia as before and hypoxic effects on the cells were verified by Western blot (supplemental figure 1D). The results did not show any difference between the control and the hypoxic condition (supplemental figure 1E).

These results show that the EPO uORF-mediated repression is released under ischemia and hypoxia along with HIF1α expression.

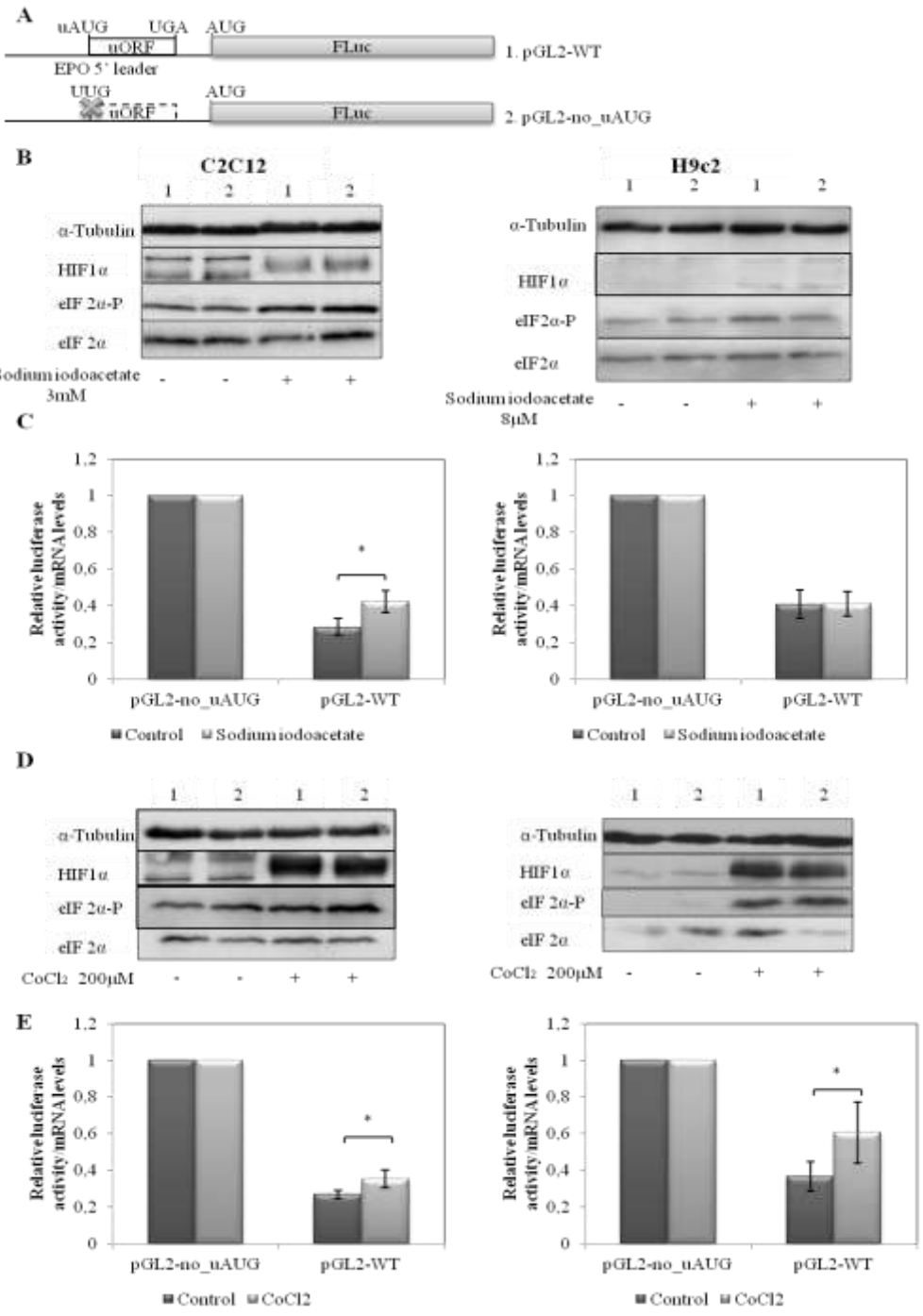

Figure 5. Hypoxia alleviate the translational repression exerted by the EPO uORF in muscle and heart cells; on the other hand, chemical ischemia has the same effect on musckle cells but does not affect EPO uORF-mediated repression in heart tissue. (A) Schematic representation of the pGL2-WT and pGL2-no_uAUG constructs. **(B)** C2C12 and H9c2 cells were transiently cotransfected with the plasmids represented in A and with the pRL-TK plasmid encoding RLuc. To mimic ischemia, 20h and 16h post-transfection, the C2C12 and H9c2 cultures were changed to fresh medium (-) or supplemented respectively with 3 mM and 8μM Sodium iodoacetate (+). Representative Western blot analysis of both cell lines extracts untreated or treated with sodium iodoacetate as indicated. Immunobloting was performed using the α-tubulin-specific antibody as loading control and the HIF1α-specific antibody, the eIF2α phosphorylated and total specific antibody to control the stress conditions. **(C)** Relative

translational efficiency of the C2C12 and H9c2 cells untreated or treated with sodium iodoacetate analyzed as described in the legend of Figure 1B. **(D)** Representative Western blot analysis of C2C12 and H9c2 extracts untreated (-) or treated (+) with 200μM of $CoCl_2$ for 6h and 15h, respectively, using the same panel of specific antibodies as before. **(E)** Cells cultured in presence or absence of $CoCl_2$ were lysed and analyzed as described in the Figure 1B.

The eIF2α phosphorylation does not determinate the EPO translational regulation

During a stress as ischemia and hypoxia the availability of competent initiation complexes decreases as a consequence of the eIF2α phosphorylation (Sonenberg & Hinnebusch 2009). However, several studies show that transcripts bearing uORFs can increase the expression of certain stress-related mRNAs despite the eIF2α phosphorylation (Dang Do et al. 2009; Barbosa & Romão 2014; Koritzinsky & Wouters 2007). Bearing this in mind, we stimulated the eIF2α phosphorylation in order to verify if the translational derepression caused by ischemia and hypoxia could be justified by the eIF2α phosphorylation. Our panel of cells was transfected with the pGL2-WT and pGL2-no_uAUG constructs and then untreated or treated with 1μM of thapsigargin (Figure 6A). The analysis was made as before and the levels of the eIF2α phosphorylation were confirmed by Western blot (Figure 6B). Figure 6C shows that the translational regulation exerted by the EPO uORF is not altered by the eIF2α phosphorylation alone. This may indicate that HIF1α expression is needed to bypass the uORF translation in response to stress as suggested from results shown in figure 6.

Additionally, we also stimulated the eIF2α phosphorylation in the presence of the EPO 3'UTR. The experimental procedures and result analysis were the same as before using the pGL2-WT, pGL2-WT-3'UTR and pGL2-no_uAUG-3'UTR constructs (supplemental figure 2A). Cells were untreated or treated with 1μM of thapsigargin and the phosphorylation levels of the eIF2α were verified by Western blot (supplemental figure 2B). Results show an increase in translation efficiency, during the eIF2α phosphorylation, when the uORF is inactivated in the presence of the 3'UTR (supplemental figure 2C).

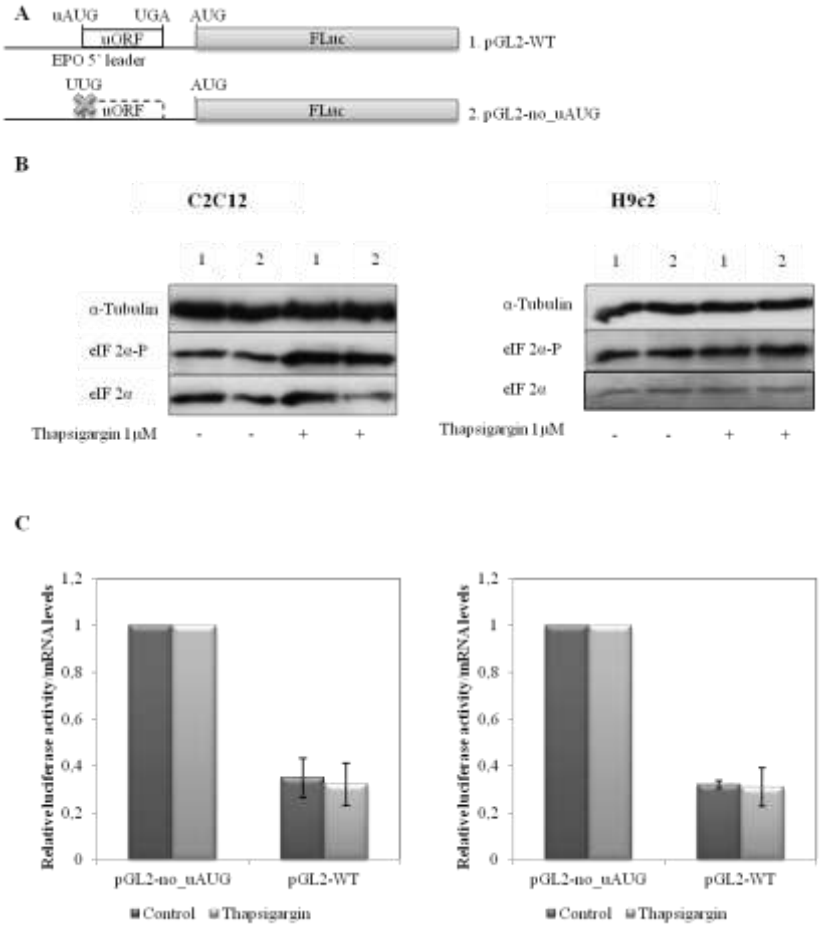

Figure 6. The eIF2α phosphorylation is not responsible for the EPO translational derepression. (A) Schematic representation of the pGL2-WT and pGL2-no_uAUG plasmids. **(B)** Representative Western blot analysis of C2C12 and H9c2 cells extracts untreated (-) or treated (+) with 1μM of thapsigargin during 20 hours to activate eIF2α kinases and induce eIF2α phosphorylation. Immunobloting was performed using the α-tubulin-specific antibody as loading control and the eIF2α phosphorylated and total specific antibody to control the stress conditions. **(C)** Relative translational efficiencies in C2C12 and H9c2 cells, untreated or treated with 1μM of thapsigargin, analyzed as described in the legend of the Figure 1B.

DISCUSSION

The 5'UTR of the human EPO compasses a 14 codon uORF that has been demonstrated to significantly inhibit the translation of the downstream ORF in different cell lines (Barbosa & Romão 2014). Here we confirmed this translation inhibition in muscle and cardiac cells (Figure 1). The preservation of this effect in such a different panel of cell lines shows the determinant role of this EPO uORF in the translational regulation.

The ribosome access to the AUG of the main ORF is mainly achieved by reinitiation after the uORF translation. Only a few ribosomes are able to bypass the uAUG due to its good context that despite the lack of the suitable nucleotide in the position +4 is good enough to be recognized most of the times (Figure 2). The uORF-encoded peptide is highly conserved among the species (Barbosa & Romão 2014), however this

small peptide does not seem to be involved in the translational repression exerted by the EPO uORF (Figure 3).

We also verified that the 3'UTR has a different influence in the two cell lines. While in C2C12 cells there is a 2-fold increase on translation efficiency in the presence of the 3'UTR, in H9c2 cells there is no effect of the 3'UTR (Figure 4). It is also noteworthy that the uORF maintains its ability to inhibit the translation in the presence of the 3'UTR in both cell lines.

Although both cells are myoblasts they revealed several differences when were subjected to the sodium iodoacetate treatment. While the C2C12 cells produced HIF1α a typical sign of oxygen deprivation, the H9c2 cells did not, showing that these cells have different strategies to overcome ischemia. On the other hand, the C2C12 cells were able to respond to ischemic stress by increasing the translation of the main ORF showing that the uORF is bypassed during this situation; however, the H9c2 cells did not (Figure 5). Under hypoxia, both cells increased the translational efficiency of the reporter mRNA and expressed HIF1α, showing that this protein may be essential for the translational regulation exerted by the EPO uORF (Figure 5). However, direct eIF2α phosphorylation by thapsigargin treatment, a stressor of the endoplasmic reticulum, did not affect reporter translational efficiency (Figure 6).

In this work we were able to demonstrate that the uORF present in the 5'UTR of the human EPO transcript is able to control translation, being the EPO uORF-mediated repression alleviated in response to some stresses specifically when the HIF1α is produced.

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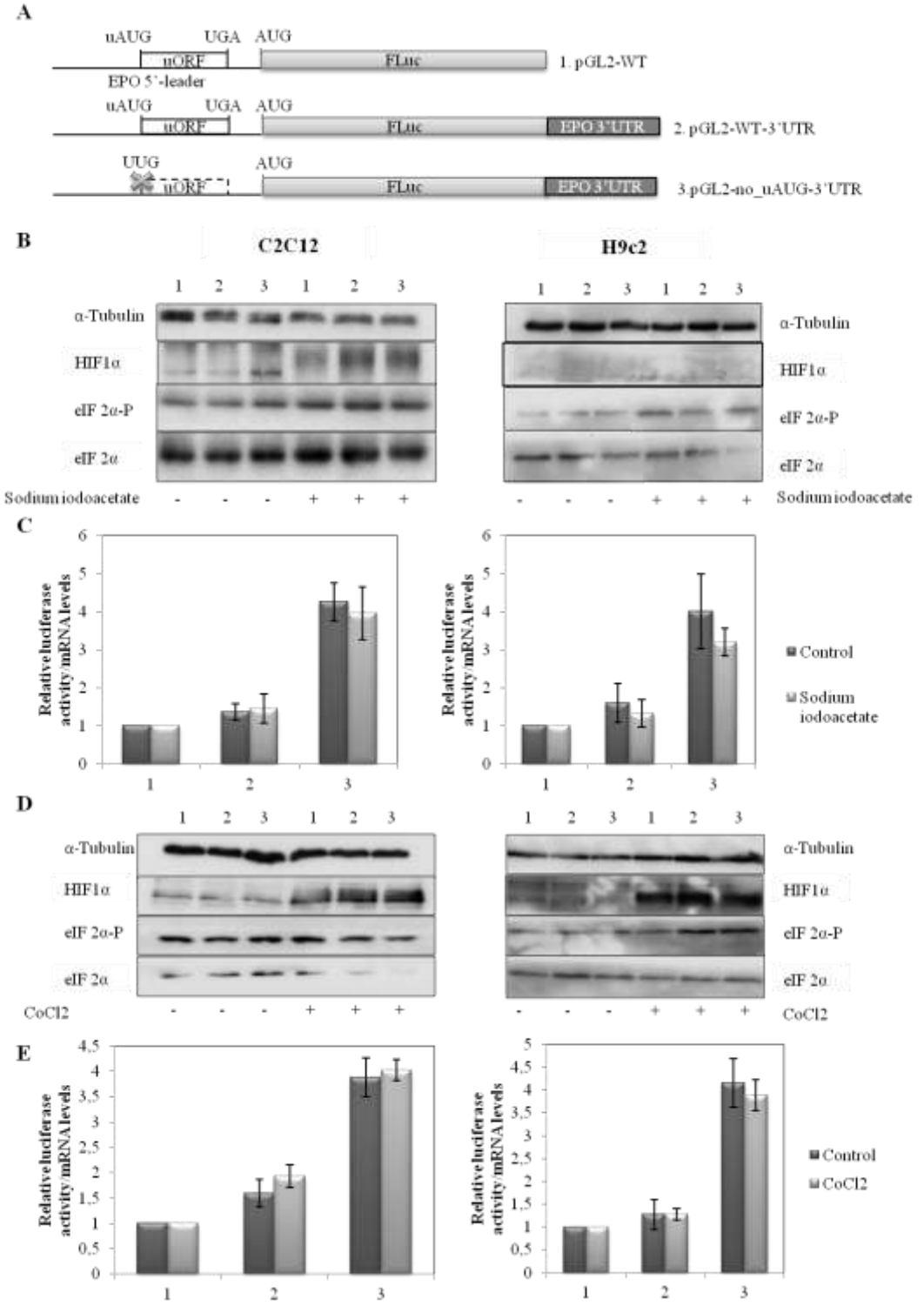
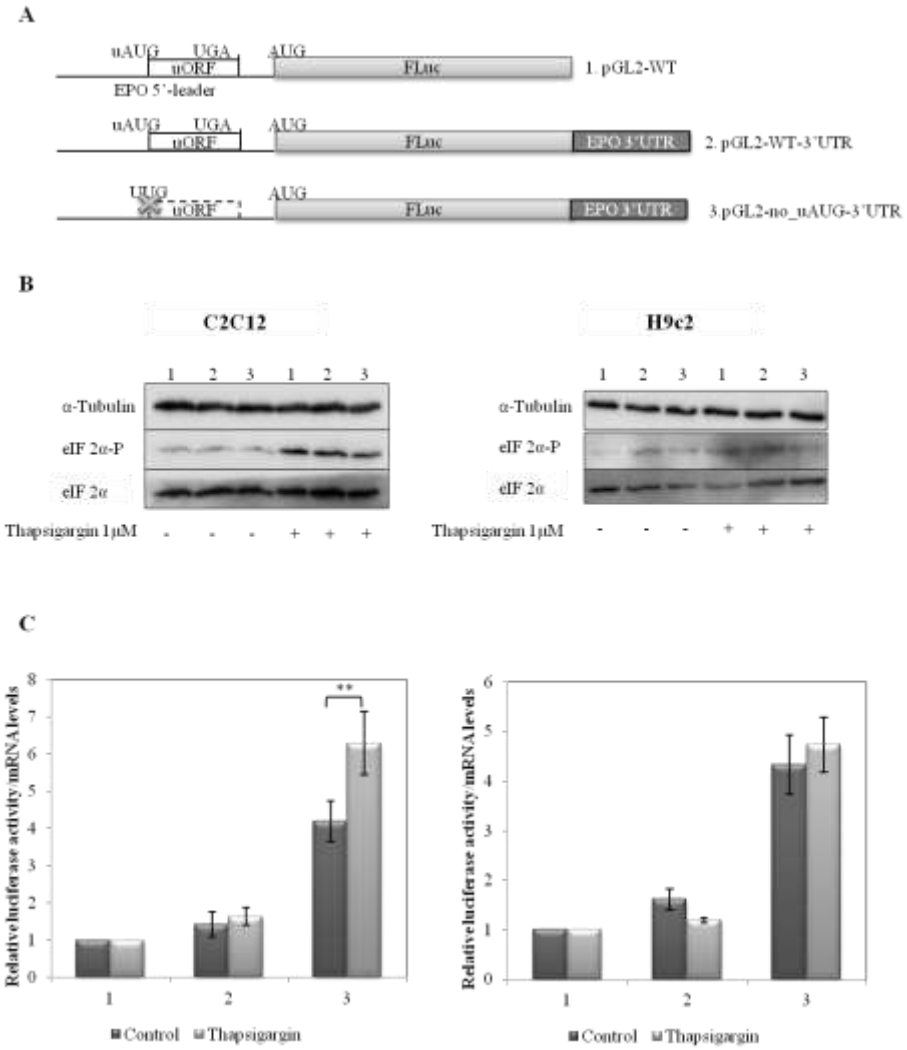
SUPPLEMENTAL FIGURES



Supplemental figure 1. The translational regulation is maintained in the presence of the EPO 3'UTR under either ischemia or hypoxia. (A) Schematic representation of the pGL2-WT (construct 1), pGL2-WT-3'UTR (construct 2) and pGL2-no_uAUG-3'UTR (construct 3) plasmids described in the figure 4A. **(B)** Representative Western blot of C2C12 and H9c2 cells untreated (-) or treated with 3mM and 8µM Sodium iodoacetate (+) respectively to mimic ischemia. Immunobloting was performed using the HIF1α-specific antibody, the eIF2α phosphorylated and total specific antibody to control the stress conditions. As loading control the α-tubulin-specific antibody was used. **(C)** Cells cultured in presence or absence of sodium iodoacetate were lysed and analyzed as described in the Figure 1B. **(D)** Representative Western blot analysis of C2C12 and H9c2 extracts untreated (-) or treated (+) with 200µM of $CoCl_2$ for 18h using the same panel of specific antibodies as before. **(E)** Relative translational expression of the C2C12 and H9c2 cells untreated or treated with 200µM of $CoCl_2$ analyzed as described in the legend of the Figure 1B.



Supplemental figure 2. In C2C12 cells, eIF2α phosphorylation increases translation when the uORF is inactivated in the presence of the EPO 3'UTR. (A) Schematic representation of the pGL2-WT (construct 1), pGL2-WT-3'UTR (construct 2) and pGL2-no_uAUG-3'UTR (construct 3) plasmids described in the figure 4A. **(B)** Representative Western blot of C2C12 and H9c2 cells untreated (-) or treated with 1μM of thapsigargin (+) to activate eIF2α kinases and induce eIF2α phosphorylation. Immunobloting was performed using the eIF2α phosphorylated and total specific antibody to control the stress conditions. As loading control the α-tubulin-specific antibody was used. **(C)** Relative translational expression of both cell cultures untreated or treated with 1μM of thapsigargin analyzed as described in the legend of the Figure 1B.