IRES-dependent regulation of FGF-2 mRNA translation in pathophysiological conditions in the mouse


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Abstract
The mRNA coding for FGF-2 (fibroblast growth factor 2), a major angiogenic factor, is translated by an IRES (internal ribosome entry site)-dependent mechanism. We have studied the role of the IRES in the regulation of FGF-2 expression in vivo, under pathophysiological conditions, by creating transgenic mice lines expressing bioluminescent bicistronic transgenes. Analysis of FGF-2 IRES activity indicates strong tissue specificity in adult brain and testis, suggesting a role of the IRES in the activation of FGF-2 expression in testis maturation and brain function. We have explored translational control of FGF-2 mRNA under diabetic hyperglycaemic conditions, as FGF-2 is implied in diabetes-related vascular complications. FGF-2 IRES is specifically activated in the aorta wall in streptozotocin-induced diabetic mice, in correlation with increased expression of endogenous FGF-2. Thus, under hyperglycaemic conditions, where cap-dependent translation is blocked, IRES activation participates in FGF-2 overexpression, which is one of the keys of diabetes-linked atherosclerosis aggravation. IRES activation under such pathophysiological conditions may involve ITAFs (IRES trans-acting factors), such as p53 or hnRNP AI (heterogeneous nuclear ribonucleoprotein AI), recently identified as inhibitory or activatory ITAFs respectively for FGF-2 IRES.

Translation initiation by the IRES (internal ribosome entry site)-dependent mechanism
Until the end of the 1980s, the unchallenged model of translation initiation in eukaryotes was the so-called ribosome scanning mechanism [1]. According to that model, ribosome recruitment occurs upon recognition of the capped 5′ mRNA end by the eukaryotic initiation factor eIF4F, followed by ribosome scanning along the RNA molecule until an initiation codon is recognized. This dogma has been discredited by the discovery of an alternative mechanism, the internal ribosome entry, involving an mRNA element, the IRES, that is present in the 5′-UTR (untranslated region) [2]. This mechanism was first discovered in uncapped picornavirus mRNAs, but, a few years later, it appeared that IRESes are also present in the capped mRNAs of several cellular genes, mostly coding for regulatory proteins, including proto-oncogenes, growth factors, apoptosis factors and angiogenesis factors [3].

This discovery raised an interesting question: why should a cellular capped mRNA be translated by a cap-independent mechanism? One hypothesis was that IRESes might allow translation under conditions where cap-dependent translation is abolished, as occurs under stress conditions and during the M phase of the cell cycle [4]. As a matter of fact, it was subsequently reported that IRESes drive translation initiation during various stress conditions such as heat shock [BiP (immunoglobulin heavy-chain binding protein) IRES], hypoxia [VEGF (vascular endothelial growth factor) IRES], amino acid starvation [cat-1 (cationic amino acid transporter 1) IRES] or apoptosis [XIAP (X-linked mammalian inhibitor of apoptosis protein), c-myc, APAF-1 (apoptotic protease-activating factor 1) and Bag-1 (Bcl2-associated athanogene) IRESes], as well as during the G2/M stage of cell cycle [ODC (ornithine decarboxylase) and PITSRE protein kinase IRESes]. This suggested a physiological function of IRESes in the control of gene expression in response to different stimuli [5]. However, the different reports on IRES activities were all performed in vitro or upon transient cell transfection and thus could not answer the question of the pathophysiological role of IRES. This led us to address the question in vivo, by creating transgenic mice lines that could be subjected to
Figure 1 | Proposed mechanism of the FGF-2 mRNA IRES regulation by p53 and hnRNPI in response to pathophysiological stimuli

The FGF-2 mRNA is schematized (5′ and coding regions). C0–C3 and A represent the five initiation codons CUG0–3 and AUG respectively. According to our data, the IRES is activated in response to pathophysiological stimuli, such as testis maturation, brain function and diabetic hyperglycaemia (see the text). Two ITAFs have been characterized, hnRNPI (AI) and p53, which have activatory and inhibitory effects on IRES activity respectively. The proposed model is that the two ITAFs have antagonistic effects: the mRNA may adopt two conformations, the first one allowing translation, and the other one generating translation blockade. Thus hnRNPI would have a chaperone or RNA-remodelling effect resulting in IRES stabilization and translated RNA conformation, whereas p53 generates formation of the translation inhibitory RNA conformation leading to IRES as well as cap-dependent translational silencing. IRES activation in response to pathophysiological stimuli could result on either hnRNPI activation or p53 inactivation, or both. This model does not rule out the involvement of additional ITAFs remaining to be discovered.

The FGF-2 mRNA, a model system for translational regulation

FGF-2, also known as basic FGF, belongs to the FGF family composed of 22 genes that are involved in the control of cell proliferation and differentiation, particularly during embryogenesis. This growth factor is a major angiogenic factor, but also a neurotrophic factor [6,7].

The various physiological roles of FGF-2 can be partly explained by the existence of different isoforms of the factor, which display different modes of action. These isoforms are translated from five in-frame start codons including four CUGs and one AUG (Figure 1) [8,9]. Their different N-terminal regions give them distinct localizations and modes of action: the CUG-initiated isoforms contain a nuclear localization sequence and are thus nuclear and have intracrine effects [10]. In contrast, the AUG-initiated isoform is cytoplasmic and secreted, and is responsible for the receptor-mediated
paracrine and autocrine effect of the growth factor. These isoforms thus have different functions; the CUG0-initiated form has a role of survival factor [9]. The CUG1-, 2- and 3-initiated forms are responsible for endothelial cell immortalization and radioresistance when constitutively expressed, whereas the AUG-initiated form can transform cells and is responsible for cell migration [11,12].

FGF-2 expression is post-translationally regulated by different elements present in its mRNA untranslated or alternatively translated regions. Regulatory elements have been identified in mRNA 5′- and 3′-untranslated or alternatively translated regions. By looking for the mechanism responsible for the choice of the alternative initiation codons, we have discovered an IRES in the FGF-2 mRNA [13]. This IRES, located in the first 176 nt of the FGF-2 mRNA leader, drives translation initiation from CUG1, 2 and 3 and AUG, whereas translation from CUG0 is exclusively cap-dependent [9,14] (Figure 1). Unexpectedly, a single IRES module is responsible for translation initiation at four distantly located codons. The IRES has been characterized by chemical and enzymatic probing, revealing two stem–loop regions and a G quartet motif which constitutes a novel IRES structural determinant [14].

The huge 3′-UTR (5724 nt) of the FGF-2 mRNA also contains regulatory elements. This 3′-UTR contains eight alternative polyadenylation sites that are used differently according to the cell type. We have identified two regulatory elements in the FGF-2 mRNA 3′-UTR: an AU-rich RNA destabilization element is present between polyadenylation sites 1 and 2, whereas a translational activator is located downstream from polyadenylation site 4 [15,16]. The 3′ translational activator is able to modulate alternative initiation of translation and its cross-talk with the IRES remains to be determined. Altogether, this 3′-UTR provides an interesting example of coupling between alternative polyadenylation, RNA destabilization and translation control.

The FGF-2 IRES has a tissue-specific activity in vivo

The FGF-2 IRES activity has been studied by the bicistronic vector strategy, in which the first and the second cistrons are the Renilla (LucR) and firefly (LucF) luciferases respectively. The sequence expected to have an IRES activity is placed between the two reporter genes. This approach is now the classical means of characterizing IRESs: in a bicistronic construct, the first cistron (LucR) is translated in a cap-dependent manner, whereas the second cistron (LucF) is IRES-dependent (controls are necessary to rule out the absence of internal promoter or splicing site in the intercistronic region) [17]. The FGF-2 IRES has been first studied in transiently transfected cells, revealing a low activity compared with that of EMCV (encephalomycarditis virus) IRES, especially in primary cells such as human skin fibroblasts [18]. However, skin fibroblast transformation by SV40 (simian virus 40) large T is accompanied by activation of FGF-2 IRES [19].

In an attempt to understand the biological significance of IRES-dependent translation, we have addressed the FGF-2 IRES regulation in vivo, by creating transgenic mice models that express the double-luciferase transgene with either the FGF-2 or the EMCV IRES in the intercistronic region. The results are really striking: in E11 (embryonic day 11) embryos, the FGF-2 IRES activity has a similar order of magnitude to that of EMCV IRES. In E16 (embryonic day 16) embryos, FGF-2 IRES activity starts to decrease in several organs, but not in brain. Finally, in adult mice, the FGF-2 IRES is weak in most organs, but remains highly active in brain and testis. In contrast, the EMCV IRES analysed in the same conditions remains active in all organs [18].

These data correspond to the first study of IRES regulation in vivo in mammals. Additional data obtained with transgenic mice lines expressing a transgene with the c-myc IRES show a drastic down-regulation of the c-myc IRES activity in adults by comparison with embryos, suggesting that IRESs from cellular mRNAs could be submitted to a strong tissue-specific regulation, not observed for the viral EMCV IRES [20].

From a physiological point of view, the FGF-2 IRES activity observed in brain and testis is consistent with the biological role of FGF-2. Indeed, this growth factor is well-known for its neurotrophic activity, and is able to promote neuron survival and differentiation [6]. With regard to testis, FGF-2 has been described for its involvement in both development and maintenance of testis function. This suggests a physiological role of the IRES in activation of FGF-2 isoform expression in very important physiological processes occurring in the central nervous system as well as in testis.

IRES-dependent activation of FGF-2 expression in diabetic mice vessels

FGF-2 is also a major angiogenic factor that is involved in physiological as well as pathological processes that affect the vascular system. In particular, FGF-2 is implicated in the diabetes-associated vascular complications of atherosclerosis: it is a potent growth factor for vascular cell types, including endothelial and smooth muscle cells, whose proliferation appears to be involved in pathological processes that are linked to vascular diseases. In addition, FGF-2 is involved in the pathogenesis of vascular dysfunction induced by hyperglycaemia, including vascular hyperpermeability and haemodynamic change, increased aortic smooth muscle growth, response to vascular injury and development of diabetic retinopathy [21].

To test the possibility of a change in FGF-2 IRES activity related to diabetes, hyperglycaemia was generated by streptozotocin treatment in the transgenic mice expressing the bicistronic double-luciferase transgene mentioned above. Interestingly, diabetes generates specific activation of the FGF-2 IRES in aorta, in correlation with an increased expression of endogenous FGF-2. Such an activation is not observed in heart, eye or brain. Aorta smooth muscle cells cultivated ex vivo also show an increased IRES activity and FGF-2 expression, whereas, in contrast, the global translation
of cellular mRNAs decreases. This indicates that IRES-dependent activation of FGF-2 mRNA translation, by generating overexpression of FGF-2 isoforms, might be an important parameter in the complications of atherosclerosis. Our data suggest that translational regulation in vessels might be a key phenomenon in the link between diabetes and atherosclerosis. Such a regulation is probably not limited to FGF-2: it has been reported that the CD36 scavenger receptor, contributing to accelerated atherosclerosis in response to glucose, is translationally activated in vascular wall macrophages [22]. Further investigation will provide information about the possible activation of other IRES-containing mRNAs such as VEGF mRNA under diabetic conditions.

**ITAFs that control the FGF-2 IRES activity**

The tissue-specific activity of the FGF-2 IRES, as well as its vessel-specific activation in a pathophysiological situation, such as diabetes, suggest the involvement of regulatory ITAFs. Several, but not all, viral and cellular mRNA IRESs require the involvement of ITAFs; the most studied ITAF is PTB (polyadenosine tract-binding protein) or hnRNP (heterogeneous nuclear ribonucleoprotein) I, an activator of the EMCV IRES [23,24]. PTB can also act in synergy with another ITAF, unr (upstream of N-ras), to activate the pro-apoptotic factor APAF-1 IRES by remodelling the APAF-1 mRNA conformation [25]. Interestingly, PTB can also inhibit the activities of unr and BiP mRNA IRESs [26,27]. c-myc, Bag-1 and XIAP IRESs are activated by proteins of the poly(rC)-binding protein family {including PCBP, poly(rC)-binding protein-1, -2 and hnRNP K} [28–30]. These reports show that ITAFs may be IRES activators as well as inhibitors, by acting as chaperones, RNA structure stabilizers or remodelling factors.

We have addressed the question of ITAFs that control FGF-2 mRNA activity by two different approaches. First, the observation that the FGF-2 IRES is up-regulated or constitutively activated in several transformed, but not primary, cells has led us to look for the putative role of a tumour suppressor in the regulation of FGF-2 mRNA translation [19]. With regard to such a hypothesis, p53 is an interesting candidate; although it is well known for its transcriptional trans-acting effect, several reports indicate that p53 has RNA-binding and annealing activities and is also involved in translational processes. The possible effect of p53 on FGF-2 expression has been assessed by using bigenic vectors containing both the effector and the target under the control of two distinct promoters [31]. This approach has demonstrated clearly that p53 is able to inhibit FGF-2 expression in a post-transcriptional manner, and that this inhibition by p53 does not involve its trans-acting domain or its transcriptional targets (Figure 1). Using in vitro experiments, we have shown that p53 inhibits mRNA translation initiation by inducing a conformational change of the FGF-2 mRNA leader region structure. Such an RNA secondary or tertiary structure inactivates the IRES as well as it blocks the cap-dependent translation initiation [32].

The second approach has been biochemical: by RNA affinity chromatography and MS/MS, we have recently identified hnRNP AI as a protein that specifically binds to the FGF-2 mRNA IRES. hnRNP AI activates translation initiation in vitro at the four IRES-dependent codons, but not at the cap-dependent one. Furthermore, sRNA (small interfering RNA)-induced knock down of hnRNP AI expression in cell culture inhibits the synthesis of the four IRES-dependent FGF-2 isoforms [33] (Figure 1).

We have thus characterized two ITAFs that are able to modulate the FGF-2 IRES activity: p53 as an inhibitory ITAF and hnRNP AI as an activatory ITAF. These two proteins have antagonistic effects on FGF-2 mRNA translation (Figure 1). The interplay between these two ITAFs and the possible implications of additional ITAFs remain to be determined, as well as the role of these ITAFs in IRES regulation under physiological conditions such as cerebral, testicular or vascular functions. ITAF involvement in IRES dysregulation generated by pathological situations and diseases, such as diabetes, atherosclerosis and cancer, also constitute a main objective of our future work. In such a perspective, the use of siRNAs in vivo to knock down these proteins, appears as an attractive and powerful perspective.

**References**


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