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Testosterone regulates FGF-2 expression during testis maturation by an IRES-dependent translational mechanism.

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ABSTRACT

Spermatogenesis is a complex process involving cell proliferation, differentiation and apoptosis. Fibroblast Growth Factor 2 (FGF-2) is involved in testicular function, but its role in spermatogenesis has not been fully documented. The control of FGF-2 expression particularly occurs at the translational level, by an internal ribosome entry site (IRES)-dependent mechanism driving the use of alternative initiation codons. To study IRES activity regulation in vivo, we have developed transgenic mice expressing a bicistronic construct coding for two luciferase genes. Here we show that the FGF-2 IRES is age-dependently activated in mouse testis, whereas EMCV or c-myc IRESs are not. Real time PCR confirms that this regulation is translational. By using immunohistological techniques, we demonstrate that FGF-2 IRES stimulation occurs in adult, but not in immature, type-A spermatogonias. This is correlated with activation of endogenous FGF-2 expression in spermatogonia, whereas FGF-2 mRNA transcription is known to decrease in adult testis. Interestingly, the FGF-2 IRES activation is triggered by testosterone and partially inhibited by siRNA directed against the androgen receptor. Two-dimensional analysis of proteins bound to the FGF-2 mRNA 5'UTR after UV cross-linking reveals that testosterone treatment correlates with the binding of several proteins. These data suggest a paracrine loop where IRES-dependent FGF-2 expression, stimulated by Sertoli cells in response to testosterone produced by Leydig cells, would in turn activate Leydig function and testosterone production. In addition, nuclear FGF-2 isoforms could be involved in an intracrine function of FGF-2 in the start of spermatogenesis, mitosis or meiosis initiation. This report demonstrates that mRNA translation regulation by an IRES-dependent mechanism participates in a physiological process.

INTRODUCTION

Fibroblast growth factor 2 (FGF-2) is involved in both development and maintenance of testis function in a broad number of species, including humans, bovines, rodents, stallions, roe deer and fishes (1-5). Evidence indicates that FGF-2 has a critical role in gametes production as well as steroids synthesis and release, which are the most important testicular functions (6-13). In immature testis, FGF-2 is mostly expressed by Sertoli cells, then expression of its mRNA is down-regulated in an age-dependent manner (14, 15). Expression of FGF2 has also been reported in germ cells. Especially, a meaningful expression has been observed in type-A spermatogonias (stem cells of the germ line lineage), pachytene spermatocytes (premeiotic germ cell) as well as spermatids (postmeiotic stage) in both the adult rat and mouse testis (3, 16).

FGF-2 belongs to a family of 22 evolutionarily conserved gene (17). The human FGF-2 mRNA is one of the most striking systems of translational regulation (18-21). Its highly structured 5' leader region contains five initiation codons, an AUG coding for a 18 kDa isoform, and four non-canonical CUGs, coding for high molecular weight isoforms (HMW) of 22, 22.5, 24 and 34 kDa (22-24). We have previously shown that translation initiation, except for the 34 kDa FGF-2 isoform, can be driven by an internal ribosome entry site (IRES) located in the mRNA 5' untranslated region, allowing translation to occur by a non classical cap-independent mechanism (25-27). In mouse, the process of alternative initiation of translation has been conserved, but the CUG-initiated forms have sizes of 21 and 22 kDa. An isoform of 30 kDa has been detected, although it has not been molecularly characterized yet in mouse as resulting from an additional translation initiation (3). These different FGF-2 isoforms have different localizations and functions; the AUG-initiated 18 kDa isoform is cytoplasmic and secreted, and acts by the classical para- or autocrine way involving a membrane receptor recognition. In contrast, the CUG-initiated HMW FGF-2 isoforms are nuclear and have an intracrine action involving interaction with nuclear partners (28). Interestingly, nuclear HMW FGF-2 has been previously detected in germ cells but not in

Sertoli cells, suggesting that these isoforms could be involved in the regulation of germ cell proliferation and differentiation (3, 29).

To study the IRES regulation and function in pathophysiological processes in vivo, we have developed transgenic mice expressing a bicistronic construct coding for luciferase genes, allowing to measure IRES activity in various organs. According to this animal model, FGF-2 IRES activity is regulated in a developmental and tissue-specific manner. In the adult, the FGF-2 IRES is mostly active in brain and testis, in contrast to the encephalomyocarditis virus (EMCV) IRES, which has a wide spectrum of activity, and to the c-myc IRES, which is inactive (30, 31). This finding suggested that the IRES could be involved in the spatio-temporal control of FGF-2 expression at the translational level in testis.

In the present study, we focused on the regulation of the FGF-2 IRES activity in testis as a function of age. Our results show that this IRES is up-regulated in adult testis. This activation, correlated with endogenous FGF-2 expression, specifically occurs in spermatogonia, thus at the start of spermatogenesis. Furthermore, hormone stimulation and siRNA targeting the androgen receptor mRNA showed that this regulation is testosterone-dependent and correlated with the binding of proteins to the FGF-2 IRES RNA.

MATERIAL AND METHODS

Plasmids and generation of transgenic mice

Plasmids and transgenic mice used in this study have been already described (30, 31). Mice carry transgenes coding for two luciferase genes, Renilla luciferase (LucR) and Firefly luciferase (LucF), both controlled by the cytomegalovirus promoter (CMV) and separated by

either the Encephalomyocarditis virus (EMCV) IRES (EMCV-tg), or FGF-2 IRES (FGF-tg), or c-myc IRES (MYC-tg), as shown in Figure 1A.

The plasmid used in the cross-linking experiments was pSCT 12V CAT containing the FGF-2 mRNA leader, as described earlier (32).

Luciferase Activity Assay

Male transgenic homozygote mice of three, six and 12 weeks old (for each strain) or four, five, eight, 10, 24, and 60 weeks old (for EMCV-Tg and FGF-2-Tg strains), were sacrificed. Testes were removed, weighted and frozen in liquid nitrogen and then stored at -80°C for later use. Analysis of luciferase activity was done as reported in Creancier et al. (30, 31).

Quantification of RNA and determination of bicistronic RNA integrity by Quantitative real-time RT-PCR.

Total RNA purification

Testes of transgenic mice of the FGF-2-Tg strain at three, six and 12 weeks post-partum, and testes of wild-type mice of 12 weeks, were treated with the SV total RNA isolation System (Promega) according to manufacturer's recommendations. An additional DNase I treatment (Ambion DNA free kit) was carried out.

Reverse transcription reaction (RT)

RT was done using 4 µg total RNA and the Reverse Transcription Core Kit (Eurogentec, Belgium). The reaction product was treated with RNase H (Invitrogen).

Primers and probes design.

Specific primers and probe for Luciferase Renilla and Firefly cDNA were designed using TaqMan® Primer & Probe Design of Primer Express® v1.5 (Mac) software (Perkin

Elmer/Applied Biosystem). The sequences of primers and probes are available as supplementary informations.

5' 6-FAM, 3' TAMRA primers and probes were purchased from Eurogentec.

As an internal control, the quantification of ribosomal 18S RNA was used (Pre-developed Taqman® Assay Reagent , 18S rRNA - Applied Biosystem).

Primers and probes efficiencies were determined in preliminary experiments.

PCR conditions.

Quantitative PCR was performed in a total reaction volume of 25 µl on a 96-well reaction plate using qPCR™ Mastermix from Eurogentec according to the manufacturer's recommendations.

Final concentrations were 300 nM for the primers and 100 nM for the probes.

The amplification conditions for Gene Amp 5700 (Applied Biosystem) consisted of an initial step of 2 min at 50°C, HotGoldStar Activation 10 min 95°C followed by 40 cycles: 15 sec 95°C, 1 min 60°C.

Normalization and quantification.

Quantification of luciferase transcripts was performed using a standard curve calibration that was created by plotting the threshold cycle (Ct) versus the known copy number for the plasmid template pCRFL in the samples. To correct differences in both RNA quality and quantity between samples, data were normalized for the corresponding ribosomal 18S RNA content.

Mouse Tissue Preparation, Immunohistochemistry, Immunofluorescence Staining, and Microscopy.

Male transgenic and wild type mice of each strain of three and six weeks old were sacrificed. Testes were removed, either frozen in tissue-teck O.C.T. compound (Cryomatrix®) and kept at -80°C for later use for the immunofluorescent staining (IF), or fixed with 4% buffered

paraformaldehyde, then processed with standard methods for paraffin sectioning and used for immunohistochemistry (IHC).

IF was performed as follows : serial cryosections of each testis were cut at 10 μm , fixed with 4% paraformaldehyde in 0,1M Phosphate buffer, pH7.4 (10 min at 4°C) and treated with 0.1% Triton X-100 in phosphate buffer saline (PBS) for 3 min at room temperature, as recommended by Kamma et al. (33). Preincubation in 3% BSA-PBS and 1% normal horse serum was done prior to incubation with various antibodies. Tissue sections were then incubated for 2 h at room temperature with each antibody tested: anti-FGF-2 (1:200), anti-hnRNP A1 (1:300), anti-luciferase firefly (1:300). Either normal rabbit IgG or without first antibody was used as negative control (all antibodies were supplied by Santa Cruz Biotechnology, except for anti-LucF antibody, provided by Europa Bioproducts Ltd). Finally, both antibodies and controls were stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit F(ab')(Sigma). Propidium iodide (Sigma) was used as counterstain.

Immunohistochemistry was performed on deparaffinized sections (5 μm) with the peroxidase-antiperoxidase technique, after a pretreatment of sections with the Target Retrieval Solution of DAKO® at 95°C for 20 min. Endogenous peroxidase activity was quenched by incubation of the sections with DAKO® blocking reagent (20 min). Subsequently, the sections were treated with The Vector® M.O.M.™ immunodetection kit, following the furnished protocol. The first antibody was incubated overnight at 4°C (1:200 for anti-LucF, and 1:200 for anti-FGF-2, 1:300 for anti-Oct-3/4 ref H-134) and counterstained with hematoxin.

Stained specimens were examined using a Leica microscope (model MPS60) or a confocal microscope. Image analysis was carried out with the DC viewer and Leica Qwin software respectively, and processed by Adobe photoshop software.

Testosterone injection in transgenic mice

Water-soluble testosterone (Sigma), dissolved in PBS (1mg/kg) or the vehicle alone were intraperitoneously injected in three weeks old FGF-2-Tg male mice. Mice were killed 24 h

after injection, testes were removed, snap-frozen in liquid nitrogen and stored at -80°C until processed.

SiRNA treatment of transgenic adult mice.

SiRNA sequences used in this study are available as supplementary informations.

The AR-siRNA recognizes a sequence conserved between human, mice and rats, located in the first coding exon of the androgen receptor. The sequence of the cont-siRNA has no significant homology with any sequence in the databases. Eight weeks-old FGF-2-Tg or EMCV-Tg mice were injected intraperitoneously daily for 10 days with 100 μl of solution of siRNA in isotonic saline solution (3 μg / mouse). Mice (three per group) were sacrificed. Testes were removed and frozen in liquid nitrogen and stored at -80°C until processed.

Gel Electrophoresis and immunoblotting.

The protocol of Lahr et al. was followed with some minor modifications. Briefly, testes samples of FGF-2-Tg mice were thawed, homogenized in SDS 2% and sonicated and boiled for 5 min under reducing conditions (1). They were separated by electrophoresis, blotted onto nitrocellulose and probed with a rabbit anti-FGF-2 anti-serum (1:500), anti-AR (1 :200) (Santa Cruz N-20. Sc816) or anti- β -actin (Sigma). The mouse anti-tubulin antibody was monoclonal (clone DM1A). Immunoreaction was detected after incubation with an anti-IgG antibody from a rabbit origin conjugated with the horse peroxidase (Amersham) and revealed by autoradiography using a chemoluminescent method (ECL; Amersham).

UV cross-linking-2D gel electrophoresis.

Cell extracts and UV cross-linking.

Total cell extracts of testis of wild type mice, three and 6 weeks old, were homogenized in a buffer containing 50 mM Tris-HCl pH 8,0; 150 mM NaCl; 2 mM EDTA pH 8,0 using a dounce homogenizer. The homogenates were centrifuged at 10,000 g for 10 minutes at 4°C and

each supernatant was collected. After quantification of total proteins (Bio Rad Dc Protein Assay) 5% glycerol was added, aliquots were snap-frozen in liquid nitrogen and stored at -80°C.

Protein extracts (8 µg) were mixed with in vitro transcribed 32P-labelled FGF2 mRNA 5'UTR (100,000 cpm) in buffer GS (5 mM HEPES-KOH pH 7,9; 30 mM KCl; 2 mM MgCl₂; 0,2 mM DTT and 4% glycerol) for 15 minutes at room temperature. Samples were UV-irradiated on ice (254 nm) using a stratalinker (Stratagene) at 0,4J/cm² at 10 cm distance. 5 units of RNase one (Promega) were then added and the reaction mixtures were incubated for 30 minutes at 37°C. The samples were analysed by 2D gel electrophoresis.

2D gel electrophoresis

The methods concerning the 2D gel electrophoresis are available as supplementary informations.

RESULTS

The FGF-2 IRES is age-dependently regulated in mouse testis.

Sexually immature (three weeks *post-partum*) or mature (six, 12 or 60 weeks *post-partum*) transgenic mice (EMCV-tg, FGF-tg, MYC-tg) carrying bicistronic transgenes LucR-IRES-LucF with the EMCV, FGF-2 or c-myc IRES, respectively, were analyzed for luciferase activity in testis (Fig. 1A and 1B). Expression of the first cistron, *Renilla* luciferase (LucR), is cap-dependent, whereas expression of the second cistron, Firefly luciferase (LucF), is IRES-dependent. LucR and LucF activities were measured, and the LucF/LucR ratio was calculated to evaluate the IRES activity (Table 1 and Fig. 2B). The EMCV IRES had a strong activity in both immature and adult testes, with a moderate but constant increase along the mouse life-time, (ratio=65 at three weeks and ratio=89 at 6 weeks, which represent only a 0.4 fold increase in LucF activity; Figure1B and Table 1). The c-myc IRES activity was quite

weak (ratio=0.7) and not significantly different between immature and mature testes. In contrast, the FGF-2 IRES presented a low activity at three weeks post-partum (ratio=3.8), but this activity was increased up to six-fold in testes of six weeks old mice (ratio=22.7), slightly decreased at 12 weeks (ratio=13) and remained stable during 60 weeks (Figure 1B and Table 1). These data suggested that testis maturation is correlated with specific activation of the FGF-2 IRES. Furthermore, this activation is specific to testis since no other organ of the male reproductive tract presented an IRES activity at any age (data not shown).

In order to demonstrate that the increase of LucF activity was due to an IRES-dependent translational mechanism and not to a transcriptional or splicing event, we carried out a separate quantification of transcripts coding for LucR from those coding for LucF, by real time PCR (34, 35). Figure 2 unambiguously shows that the amount of LucF mRNA is equivalent to LucR mRNA at the different ages tested, ruling out the possibility that a cryptic promoter or a spurious splicing event could have led to expression of monocistronic LucF mRNA.

As shown in Fig. 2B and Table 1, LucR activity reflects the bicistronic mRNA level that increases in adult compared to immature testis. In contrast, the LucF activity increase, 6 fold superior to that of LucR (Fig. 1B), cannot be explained by the bicistronic mRNA increase and results from an IRES-dependent mechanism.

Figure 1

Table 1

Figure 2

Activation of FGF-2 IRES specifically occurs in spermatogonias of adult testis. In order to know which testicular cells expressed LucF *in vivo*, frozen sections of fresh testes of FGF-Tg mouse were analyzed at three weeks or six weeks *post-partum* by immunohistofluorescent staining with anti-LucF specific antibodies (Fig. 1C). LucF expression was detected in six weeks testes only, in concordance with data obtained in Fig.

1B. Furthermore, the LucF staining appeared in a specific cell type located in the periphery of the seminiferous tubule. Experiments performed by immunohistochemistry revealed the same specific staining (Fig. 1D). In contrast, immunofluorescent staining of EMCV-Tg mice testes showed a completely different localization of LucF expression: a strong staining was detected in blood vessels and interstitial cells, in both young and adult testes (Fig. 1C, 6 wks: right panel; 3 wks: not shown). In contrast, no LucF expression was observed at any age in the testes of c-myc-Tg (not shown).

The peripheral localization LucF expression in the seminiferous tubules suggested that it could concern spermatogonia, the germ line stem cells. To confirm this hypothesis, detection of hnRNPAI and Oct-3/4, two proteins specifically expressed in spermatogonia (33, 36), was performed and clearly showed that the LucF expression occurs in spermatogonia (Fig. 1C and 1D). In addition, the stained cells in FGF-2-Tg adult testes were morphologically identified as type-A spermatogonias. These cells presented a characteristic elliptical shape with a flat cellular surface resting directly on the basement membrane, and an apical surface facing the seminiferous epithelium (33, 37). These data indicated that the FGF-2 IRES, but not the EMCV IRES, is specifically activated in spermatogonia of adult testis.

Expression of endogenous FGF is spermatogonia-specific in adult testis. To analyze the correlation between IRES activation and regulation of FGF-2 expression, the detection of endogenous FGF-2 in young and mature testes was performed by immunofluorescent and immunohistochemical staining with anti-FGF-2 antibody, as well as by Western blot (Fig. 1 and 3).

Immunofluorescence analysis showed, in the immature testis at three weeks, a strong fluorescent staining of FGF-2 localized in the interstitial compartment and corresponding to the Leydig cells (Fig. 1C) (38). This interstitial labelling disappeared in the adult testis, whereas FGF-2 expression clearly appeared in spermatogonias, (Fig 1C and 1D). The specific staining described above was absent in all controls (not shown).

By Western blot analysis, the expression level of the 18, 21 and 22 kDa isoforms of FGF-2 decreased between 3 and 6 weeks in the testes, whereas a 30 kDa isoform appeared and increased up to 24 weeks (Fig. 3A and B). Although this isoform has not been molecularly described in mouse, it could correspond to the previously described human 34 kDa and rat 30 kDa FGF-2 (1, 3).

In conclusion, these data indicate that expression of endogenous FGF-2 is regulated in a cell type specific manner during testis maturation. The global FGF-2 down-regulation observed by Western blot in adult testis occurs at the mRNA level, as previously described, and probably reflects the strong decrease of FGF-2 expression observed in the interstitial compartment (27). In parallel, FGF-2 expression appears in germ cells. This up-regulation is not detectable by Western as it is masked by the concomitant decrease in the interstitial compartment.

Interestingly, FGF-2 up-regulation in germ cells fully correlates with IRES activation observed in Figure 1B, suggesting that this cell-specific increase of FGF-2 expression in adult testis occurs by the IRES-dependent translation mechanism.

Figure 3

FGF-2 IRES activation in adult mice spermatogonia is testosterone-dependent.

Testosterone has an essential contribution in the initiation of adult spermatogenesis (11, 39). In order to test whether testosterone can influence the FGF-2 IRES activity, we treated transgenic mice at three weeks post-partum with a single injection of testosterone. A five fold increase of the IRES activity in response to testosterone stimulation was observed, almost reaching the level observed in mature testis (Fig. 4A, left). This increase of LucF/LucR ratio results from LucF activation whereas LucR remains stable (Fig. 4A, middle left). Western blot with anti-FGF-2 antibody revealed an increase of the FGF-2 30 kDa band but not of the 22, 21 and 18 kDa isoforms (Fig. 4A, right). However, this result reflects FGF-2 expression in whole testis and might mask a regulation occurring specifically in spermatogonia, as seen in

Fig. 3. RT PCR analysis indicated no increase of the transgenic transcript (Fig. 4A, middle right). Taken together, these results showed that the FGF-2 IRES is activated in three weeks immature testis in response to testosterone stimulation, suggesting that the activity observed at 6 weeks is due to testosterone stimulation.

As a complementary approach we tried to block the action of testosterone in adult testis (at six weeks) by using a siRNA targeted against the androgen receptor (AR). AR-siRNA and cont-siRNA (negative control) were daily administrated by intraperitoneal injection during ten days, according to a recently validated protocol (Cabon et al. unpublished results). AR depletion was confirmed by immunoblot (Fig. 4B, right panel) and immunohistochemistry (not shown). A 20% decrease of the FGF-2 IRES was detected whereas no significant change of the EMCV IRES activity occurred (Fig. 4B, left panel).

Endogenous FGF-2 expression was also down-regulated in mice treated with AR-siRNA (figure 4B, right panel). Interestingly, this decrease mostly concerned high molecular weight 21 and 22 kDa FGF-2 isoforms.

Figure 4

The pattern of protein bound to the FGF-2 mRNA 5' UTR differs between young and adult testes. Testis extracts of three and six weeks-old mice were used for cross-linking experiments with an RNA probe corresponding to the FGF-2 5' UTR RNA (containing the IRES), and analysed by two-dimensional gel electrophoresis. Figure 4C shows a different profile of testicular proteins cross-linked to the FGF-2 RNA at three or six weeks. Interestingly, an identical profile of the adult testis was found in testis of immature mice 24 hours after treatment with testosterone, with multiple spots appearing between 37 and 120 kDa. Several bands are also present in young testis but absent in adult testis or young testis treated with testosterone (asterisks, figure 4C). These results indicate a correlation between changes of protein binding to the 5' UTR RNA and increase of FGF-2 IRES activity.

DISCUSSION

The present report describes one of the first IRES-mediated regulations of gene expression in a physiological process. It is not surprising that such a regulation has been found in testis, because this organ shows a wide spectrum of mechanisms of gene expression control, particularly post-transcriptional and translational mechanisms. Indeed, spermatogenesis in adult mammals depends on a precise orchestration of spermatogonia cell differentiation.

These data allow us to propose a scheme of FGF-2 gene regulation by testosterone in adult testis. Translational activation of FGF-2 expression by the IRES-dependent mechanism results in FGF-2 expression in spermatogonia. As FGF-2 is crucial at the onset of spermatogenesis, the IRES-dependent translational mechanism appears here, in the absence of FGF-2 produced by the paracrine way (which occurs in immature testis), as a mean to trigger spermatogonia proliferation and/or differentiation towards the spermatogenesis process (40).

A novel finding provided by our study is the hormonal control of the FGF-2 IRES activity. In the immature testis, FSH is sufficient to support spermatogenesis, while in adult this process is controlled by testosterone produced by Leydig cells (39, 41). A number of studies provide evidence that in the adult testis, testosterone is essential for initiation of spermatogonial divisions and differentiation, while FSH may provide conditions favorable to spermatid maturation (4, 11, 39). In the present report we show that testosterone triggers activation of the FGF-2 IRES. Measurement of luciferase activity in testicular extracts of three, four, five and six weeks old transgenic mice reveals that FGF-2 IRES activity does not increase gradually. It is induced between five and six weeks post-partum (data not shown) in correlation with the emergence of Leydig cell function (42). The testosterone-dependent regulation of FGF-2 IRES is fully consistent with the IRES activation process observed in adult but not in immature spermatogonia. In addition, it has been shown that FGF-2 expressed by germ line cells induces testosterone production by Leydig cells (43). Thus the

IRES would be implicated in the emergence of a paracrine loop leading to spermatogenesis in mature testis.

Furthermore, AR depletion using siRNA was able to partially suppress the IRES activation by 20%. This partial effect does not fully correlate with the 70% efficiency of AR depletion. However an indirect effect (ie ITAF activation or inhibition) could explain these different kinetics. This effect is nevertheless significant and specific to the FGF-2 IRES, as it is not observed with EMCV IRES. AR, although pivotal for adult spermatogenesis, is not expressed by spermatogonia, but by Sertoli cells (44). Thus testosterone presumably acts indirectly, by triggering in Sertoli cells expression of genes (growth factors, cell-cell communication proteins...) able to stimulate the FGF-2 mRNA translation by the IRES-dependent mechanism (45, 46). An alternative mechanism could be that this FGF-2 activation would be mediated by non genomic effects of testosterone involving a membrane receptor.

Another feature of FGF-2 expression by spermatogonia is the synthesis of FGF-2 high molecular weight isoforms. Expression of such isoforms in germ cells has been described by previous reports, and we show here that the AR siRNA blocks expression of the 22 kDa molecular weight isoform) (1, 3, 8, 29). In addition, we observe expression of a 30 kDa FGF-2 isoform in adult but not immature testis, in concordance with the literature (1, 3). Thus we can hypothesize that, in addition to expression of secreted FGF-2 leading to a paracrine loop, expression of HMW nuclear FGF-2 in response to testosterone might have a role in the initiation of spermatogenesis by an intracrine process (47).

Regulation of the FGF-2 IRES activity involves the participation of regulatory proteins, IRES trans-acting factors (ITAFs), acting as repressors or activators: we have recently shown that this IRES is negatively regulated by p53 while activated by HnRNPA1 (48-50). Distinct testis proteins bind to the FGF-2 IRES-containing RNA at three versus six weeks. Further investigation will tell us whether such proteins bound to FGF-2 mRNA in mature testis but also in the testes of 3 weeks-old mice treated with testosterone, could be ITAFs. ITAFs, especially hnRNPA1 which is specifically expressed in spermatogonia, could be induced, activated or translocated in response to a stimulus coming from Sertoli cells. This would lead

to a protein complex formation on the IRES, called "IRESome", which would activate the IRES and increase FGF-2 expression. Traditionally, the translational control is thought to play a central role in the meiotic stages of spermatogenesis, but little is known about translational control during mitosis occurring in the pre-meiotic stages. Actually, FGF-2 may act as a mitogenic factor but also as a differentiation factor (17, 51). During spermatogenesis, the IRES-dependent regulation in spermatogonia may allow FGF-2 synthesis during mitosis, when cap-dependent translation is inhibited (52, 53). This might be a reason to explain the detection of IRES activity in spermatogonia A but not in other types of germ cells. Indeed, only spermatogonia are able to reach mitosis. Alternatively FGF-2, especially nuclear high molecular weight isoforms, could participate in meiosis initiation.

Very little information about the relevance of IRES-dependent translation has been available up to now. An interesting study describes that IRES-dependent translation of egg-laying hormone is activated in *Aplysia californica* bag cell neurons after an afterdischarge, the physiological trigger for egg-laying (54). Very recently, it has been shown that the utrophin A 5'-untranslated region confers internal ribosome entry site-mediated translational control during regeneration of skeletal muscle fibers (55). Thus the finding of IRES-dependent regulation in a crucial process such as spermatogenesis, as well as these recent reports, highlights the relevance of cellular IRESs for the control of gene expression in various pathophysiological process.

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FIGURE LEGENDS

Figure 1. The FGF-2 IRES is specifically activated in mouse adult testis spermatogonia in correlation with FGF-2 endogenous expression.

A) Schematic representation of the bicistronic mRNA expressed by the transgenic mice. Transcription of this bicistronic cassette is under the control of the CMV promoter. It expresses the renilla luciferase in a cap-dependent manner and the firefly luciferase in an IRES-dependent manner. FGF-2, c-myc or EMCV IRES is located between the two cistrons.

B) Luciferase activities were measured in testis extracts of young (3 weeks) or adult (6 weeks) transgenic mice, and IRES activities calculated as the $(\text{LucF}/\text{LucR}) \times 100$ ratio. For each IRES, data are expressed relatively to the value of the 3 weeks testis ratio. The results corresponding to a representative experiment (repeated at least 3 times) are presented. Data are given as the mean \pm S.E. of the results obtained from three transgenic mice of each strain in each time point.

C) Immunofluorescence detection with a confocal microscope (enlargement 50 fold) of LucF, FGF-2 or HnRNPAI (spermatogonia specific marker) was performed using anti-LucF, anti-FGF-2 or anti-HnRNPAI rabbit polyclonal antibody on cryostat sections of 3 or 6 weeks testes of transgenic mice expressing a bicistronic mRNA with the FGF-2 IRES. Immunofluorescence is superposed with propidium iodure staining performed in parallel to detect the different testis cell types. On the right, immunofluorescence staining was performed on transgenic mouse testis (6 weeks) expressing a bicistronic transgene containing the EMCV IRES.

D) Immunohistological staining of 6 weeks deparaffinized testis sections (5 μm) with the peroxidase-antiperoxidase technique, with anti-oct 3/4 (spermatogonia specific marker), anti-FGF-2 or anti LucF antibody.

Figure 2. Increase in LucF activity is due to a translational control.

- A) The amount of bicistronic mRNA was measured from transgenic mice (FGF-2 IRES) testicular extracts by reverse transcription and real time PCR using specific probes for each cistron. A) The graph shows the mean \pm S.E. of the abundance, individually measured for each luciferase transcript. At all ages, quantities measured for LucF transcript are equivalent to LucR transcript, confirming the integrity of the bicistronic transcript and the absence of cryptic promoter in the intercistronic region. This corresponds to representative experiments repeated 3 times, with groups of 3 to 5 mice for each point.
- B) Measurement of the corresponding Luc R and LucF activities. The LucF/LucR ratio reflects the IRES activity (see Fig. 1B).

Figure 3. Western blot analysis of endogenous FGF-2 expression as a function of age.

- A) Western blot analysis of 3 weeks to 24 weeks FGF-tg testis extracts using FGF-2 antibody. The molecular weights of the different FGF-2 mouse isoforms are indicated.
- B) Quantification of the FGF-2 isoforms detected in Fig. 3A, after normalization to β -actin.

Figure 4. FGF-2 IRES regulation by testosterone and anti-androgen receptor siRNA.

A) Three weeks-old transgenic male mice were treated with a single testosterone injection of 1mg /kg of body weight diluted in PBS. Mice were sacrificed 24 h after injection and testes were immediately dissected and prepared for the luciferase activity assay. The ratios of (LucF/Luc R) \times 100 of young or adult mice injected with PBS were taken as controls.

Left panel: IRES activities in testes of young mice treated with testosterone (3W+T) and of adult mice without treatment (6W) are expressed relatively to the untreated young mice control (3W), considered as the basal level of IRES activity.

Middle left panel: Measurement of luciferase activities in testes of three weeks mice treated or not with testosterone.

Middle right panel: total RNA was purified from testis, cDNA was obtained by reverse

transcription and expression of the two cistron LucR and LucF measured by real time PCR using two distinct primer pairs.

Right panel: Western blot detection of the endogenous endogenous FGF-2 expression with in testes from 3 weeks-old mice untreated or treated with testosterone. β -actin expression was used as a loading control.

B) Androgen receptor was silenced using an AR-siRNA which specifically targets the first coding exon of the androgen receptor gene. Transgenic adult mice expressing transgenes with FGF-2 or EMCV IRES were treated for ten days starting on six weeks of age by intraperitoneal siRNA injection. IRES activities, measured as above, are expressed as percent of control (cont-siRNA). Data are the mean \pm S.E. of three mice by point * $P < 0.05$ (left panel). AR, FGF-2 and tubulin (loading control) protein levels were analyzed by western blot in homogenates of testes from each siRNA-treated mice. The right panel shows a representative result obtain on a control- or AR-siRNA treated mouse testis.

C) UV cross-linking of testis proteins bound to the FGF-2 mRNA 5' UTR. An *in vitro* transcribed RNA probe (100 000 cpm) corresponding to the FGF-2 mRNA leader sequence (containing the IRES) was incubated with testicular extracts. After UV irradiation, samples were treated with RNase One, and analyzed by two-dimensional gel electrophoresis. Asterisks point to proteins found in immature testis but not in adult testis or in immature testis treated with testosterone. Light arrows indicate the proteins cross-linked to the FGF-2 IRES in adult and testosterone-treated pubertal testes. Heavy arrow indicates the only protein bound to the FGF-2 mRNA 5' UTR in testicular extracts of adult mice but not in immature testis with or without testosterone treatment. Molecular weight markers are shown.

Table 1

	weeks <i>p p</i>	<i>Renilla</i> AU (10 ³) / mg tissue	Firefly AU (10 ³) / mg tissue	(F / R)*100
EMCV	3	146.14 ± 3.91	95.80 ± 22.93	65.6 ± 2.1
	6	164.02 ± 85.15	147.18 ± 94.29	89.7 ± 0.24
	12	122.30 ± 12.74	171.25 ± 20.97	140.0 ± 4.28
	60	130.17 ± 6.54	170.52 ± 10.77	131.0 ± 2.47
FGF-2	3	13.40 ± 1.72	0.51 ± 0.03	3.8 ± 0.4
	6	41.71 ± 4.35	9.47 ± 0.57	22.7 ± 4.1
	12	42.31 ± 2.23	6.66 ± 0.42	15.8 ± 0.49
	60	84.27 ± 1.30	10.9 ± 1.41	13.0 ± 1.47
c-myc	3	22.53 ± 4.58	0.15 ± 0.09	0.7 ± 0.4
	6	17.62 ± 3.71	0.17 ± 0.07	1.0 ± 0.3
	12	37.92 ± 10.96	0.40 ± 0.16	1.1 ± 0.2

Testicular extracts of each transgenic mice strain described in Fig. 1A were analysed for *Renilla* (R) or *Firefly* (F) *Luciferase* activity. Each value represents the means ± SE of measurements in three mice.

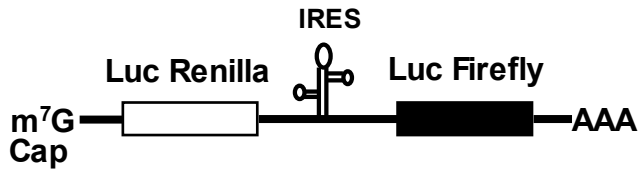
p p = post-partum

AU= Arbitrary Units

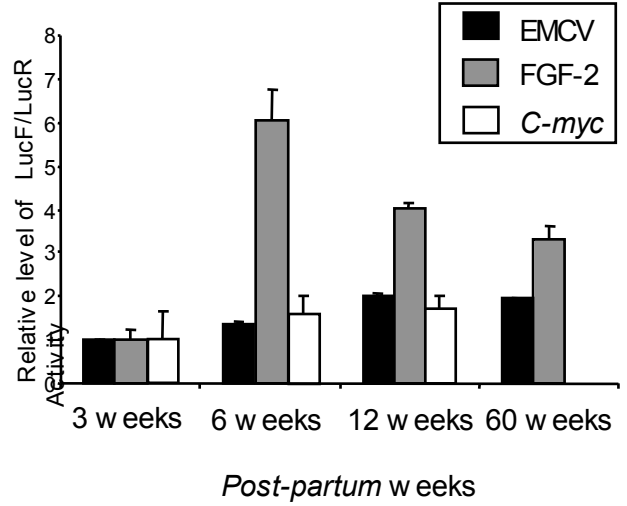
Figure 1

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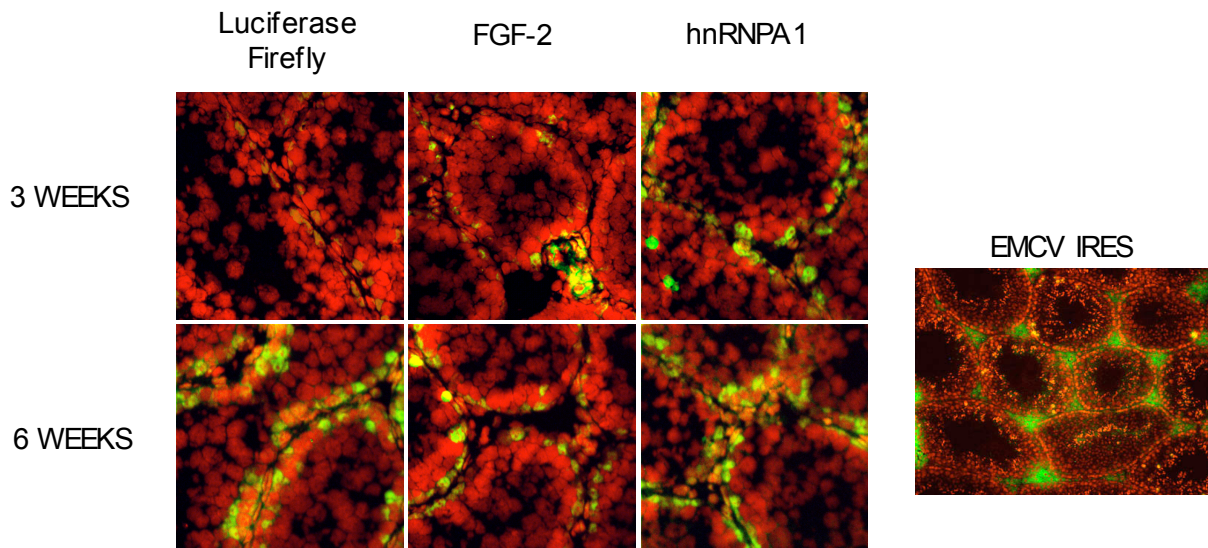
A) Bicistronic mRNA



B) FGF-2 IRES activity



C)



D)

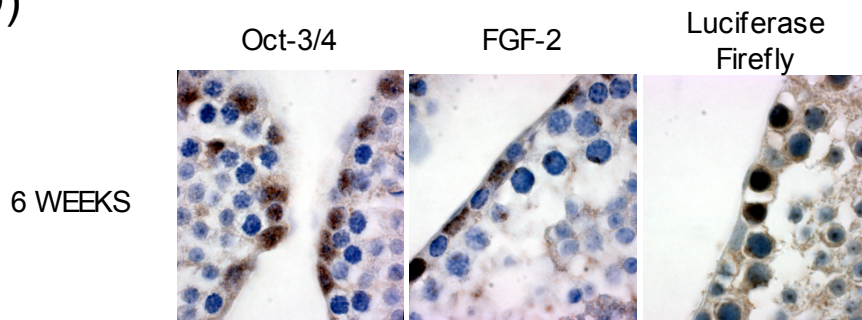
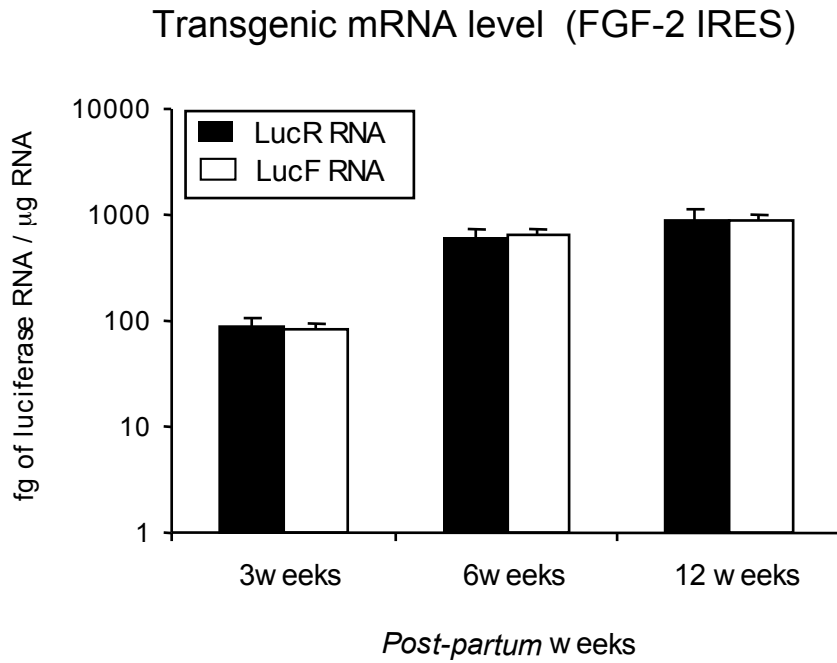


Figure 2

A)



B)

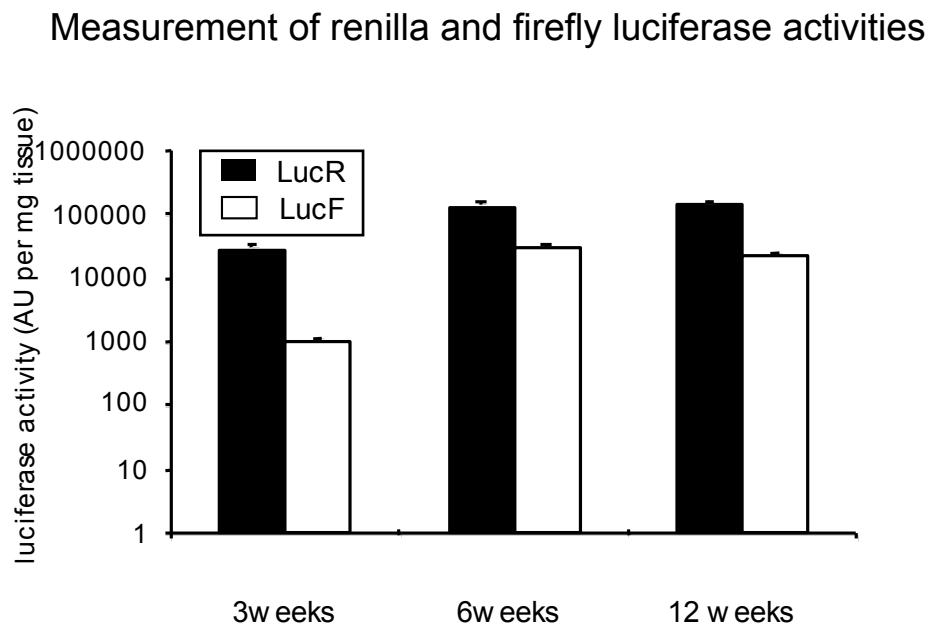
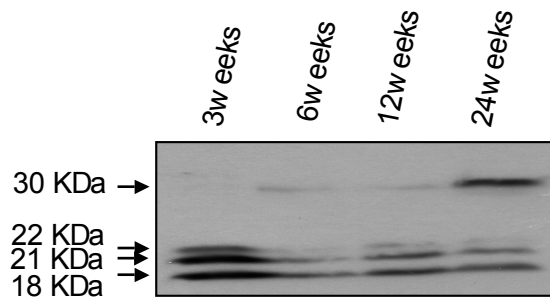


Figure 3

A) Western Blot (anti-FGF-2 antibody)



B) FGF-2 isoforms quantification

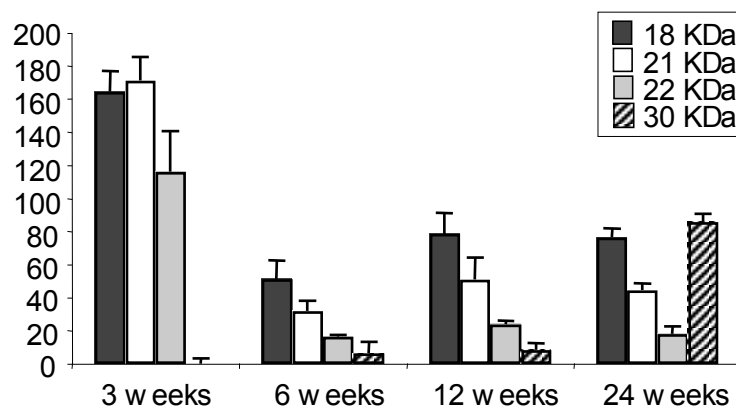
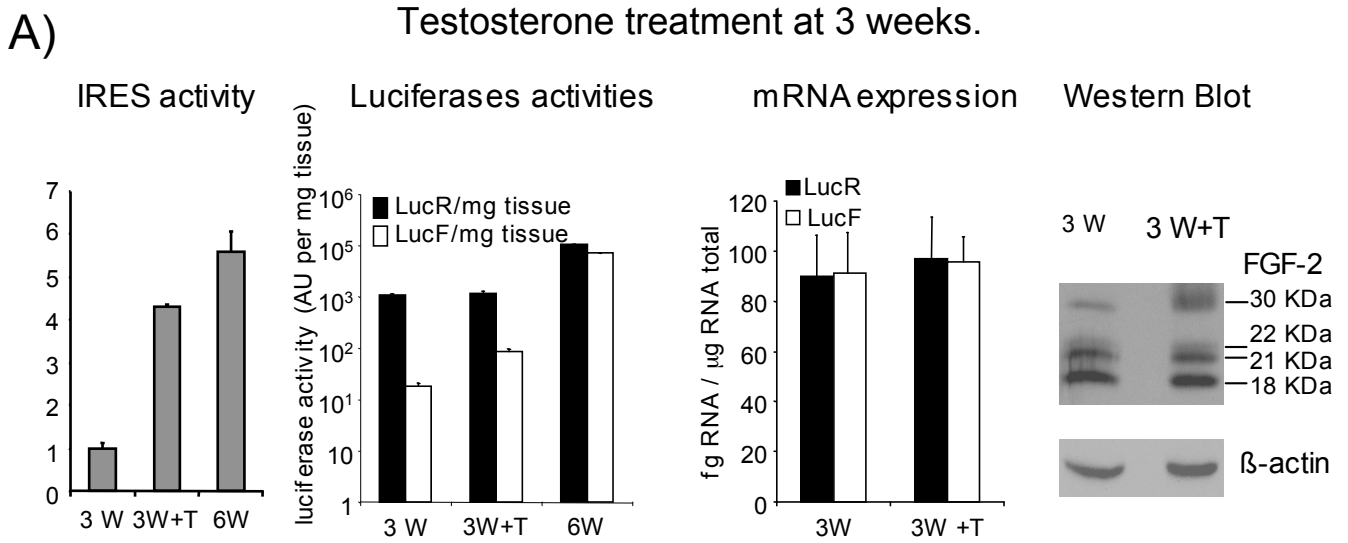
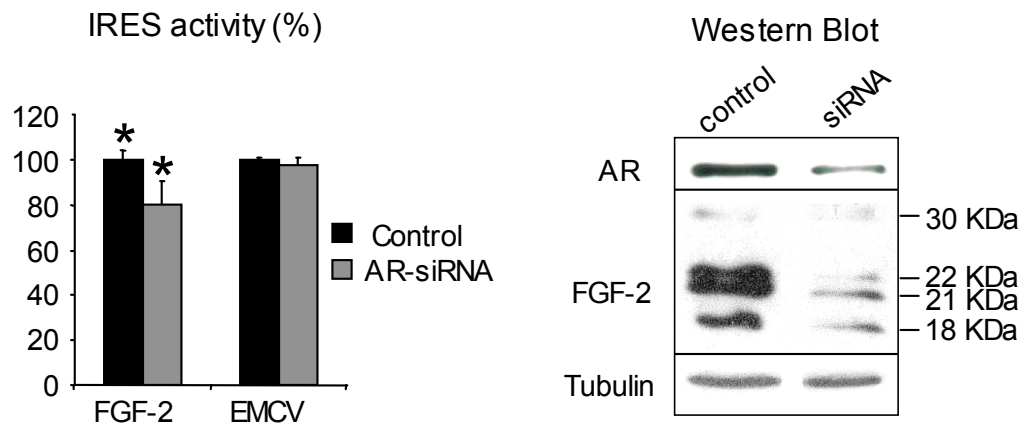


Figure 4

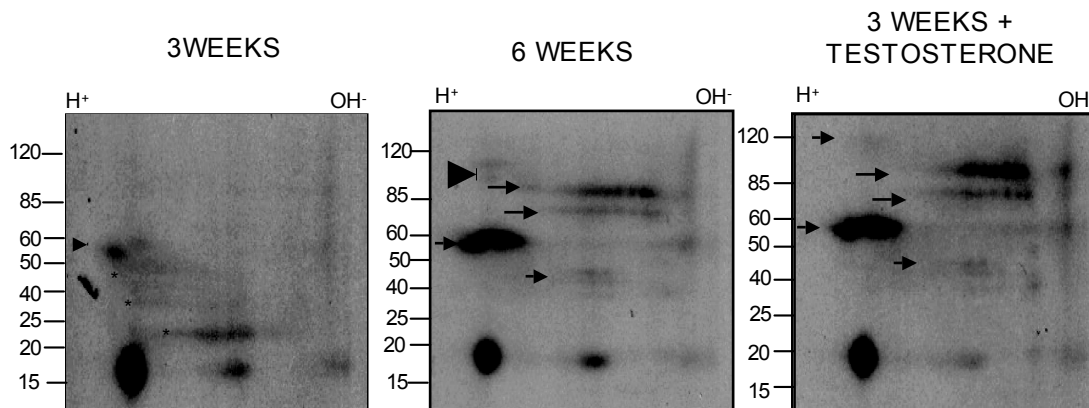
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B) Anti-androgen receptor siRNA treatment at six weeks.



C) Testis proteins bound to FGF-2 mRNA 5'UTR.



Gonzalez et al, diagram

