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DEVELOPMENT- AND AGE-DEPENDENT DOWN REGULATION OF *Fmr-1* GENE EXPRESSION IS CORRELATED WITH DECLINED SP-1 AND INCREASED USF1/USF2 INTERACTIONS TO *Fmr-1* PROMOTER IN THE BRAIN OF MALE MICE

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Abstract

Fmr-1 gene plays important role in the regulation of synaptic plasticity related gene expression and thereby cognitive function of the brain. Information on its expression as a function of postnatal brain development and aging is lacking. Therefore, aim of the present study was to evaluate the expression pattern of the *Fmr-1* gene in the cerebral cortex of postnatal day-0 to day-16 during postnatal development and young, adult and old male mice. Our Western blotting and RT-PCR data revealed that expression of *Fmr-1* mRNA transcript and FMRP was down regulated in the cerebral cortex with increasing postnatal developmental period and age. To find out whether the above age-dependent decrease in the level of *Fmr*-1 gene expression was because of alterations in in its transcriptional machinery at the levels of interaction of the transcription factors Sp-1 and USF1/USF2 (E-box) to the *Fmr-1* promoter, we used electrophoretic mobility shift assay (EMSA) technique. Our EMSA data indicated that the above postnatal developmental- and age-dependent decline in the *Fmr*-1 expression was correlated with decreased interaction of Sp1with its cognate *Fmr-1* promoter sequence whereas it was correlated with increased interaction of USF1/USF2 with its cognate *Fmr*-1 promoter sequence. Our results demonstrate that the expression of *Fmr-1* gene is positively correlated with Sp1-*Fmr-1* promoter interaction while, it is negatively correlated with USF1/USF2-*Fmr-1* promoter interaction with increasing develop-mental period and aging. Our findings on alterations in *Fmr*-1 expression may serve as one of the molecular mechanisms leading to age-dependent alterations in the brain functions.

Keywords: *Fmr-1*, FMRP, Transcription factors, Sp-1, USF1/USF2, Aging, Brain aging

Abbreviations:
FXS- Fragile X syndrome
FXTAS- Fragile X mental retardation associated tremor and ataxia syndrome
FMRP- Fragile X Mental Retardation Protein
mGluR- Metabotropic Glutamate Receptor group 1
EMSA- Electrophoretic Mobility Shift Assay
USF1/USF2- Upstream Stimulatory Factor 1/2
Sp1- Specificity protein 1

Introduction

Aging affects variety of brain functions including attention, speech, sleep, decision making, cognition, working and long-term memory. Many studies with rodent models show that old age exhibits an increased anxiety, decline in cognition and motivation, decreased interest in novel tasks, and motor disabilities (Kelleher et al. 2004). These age related changes are similar in humans also (Squire and Wixted 2011). Apart from aging, brain function is compromised due to several age dependent neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Fragile X mental retardation associated tremor and ataxia syndrome (FXTAS) and certain neurological disorders. Fragile X syndrome (FXS) is the most common inherited cause of mental retardation and it predominately affects males. One in every 4000 males and one in every 6000 females of all races and ethnic groups suffer from FXS related disorders (Coffee et al. 2009). Fragile X patients show a progressive decline in cognitive function, aberrant behavior including anxiety, mood alterations, and dementia while conditions deteriorate more severely in older males (Jacquemont et al. 2004). FXS is caused due to amplification of trinucleotide repeat CGG (>50-200) in 5'-untranslated region (5'-UTR) of the FMR1 gene. The amplification is followed by hypermethylation of CpG island in 5'-UTR and promoter region of the FMR1 gene that leads to transcriptional silencing of the gene and thereby absence of its encoded protein called Fragile X Mental Retardation Protein (FMRP). The transcriptional silencing or mutation in FMR1 causes several neurodegenerative disorders which includes several neurological paradigms like intention tremor, gait ataxia, progressive supranuclear palsy-like phenotype, Parkinsonism, FXTAS and dysautosomnia among males (Fraint et al. 2014; Grigsby 2016; Hagerman et al. 2001). FXS is also the most common cause of autism with increased rate in older individuals with FXS compared to the younger ones (Harris et al. 2008). The pre-mutation carriers express 2–8 times more FMR1 mRNA, which form aggregates and ultimately causes RNA toxicity. This RNA toxicity, combined with mitochondrial dysfunction (Napoli et al. 2011) causes the emergence of FXTAS symptoms typically after age of 50 (Schneider et al. 2013). A recent study showed that greater proportion of adult male carriers of the FXTAS endorsed subjective memory complaints compared to controls (Birch et al. 2016).

FMRP, an RNA binding protein, is involved in several steps of mRNA metabolism, nucleocytoplasmic trafficking in pre- and postsynaptic terminals, translational control and transport of multiple proteins that are important for learning and memory and the development of cortical circuits (Till 2010). In the absence of FMRP, the equilibrium of mRNP normally containing FMRP is perturbed resulting in deregulation of the expression and localization of a subset target mRNAs [13]. The absence of FMRP causes defects in the development, maintenance and plasticity of the neuronal network and its connectivity due to abnormal dendritic spines and synaptic transmission which are commonly reported in the brain of FXS individuals and *Fmr*-1 knockout mice (Santos et al. 2014). Recent report from Borek et al., (2016) suggests

that FMRP regulates synaptic vesicle dynamics thereby supporting the role of FMRP in presynaptic functions (Broek et al. 2016). Absence or decreased of FMRP synthesis at the synapse results in the prevalence of immature dendritic spines which are the predominant sites of excitatory synapse formation (He and Portera-Cailliau 2013). FMRP plays a crucial role in control of the synthesis of downstream proteins of the group 1 metabotropic glutamate receptor (mGluR) *via* inhibition of mGluR-LTD (Niere et al. 2012). Abandoned FMRP leads to up-regulated downstream effectors of group 1 metabotropic glutamate receptors (mGluR1/R5) and is associated with abnormal dendritic spine morphology indicative of insufficient pruning of unnecessary synaptic connections during development. Thus, *Fmr*-1 gene expression plays a critical role in the normal brain development and its expression should be precisely regulated to prevent disease (Schneider et al. 2013; Waung and Huber 2009).

Several *trans*- acting factors which are actively involved in regulation of *Fmr*-1 transcriptional activity involves upstream stimulatory factor 1 and 2 (USF1 and USF2), nuclear respiratory factor 1and 2 (Nrf1 and Nrf2), specificity protein 1 (Sp1), AP2α and cAMP response element binding protein (CREB) (Kumari et al. 2005; Kumari and Usdin 2001; Lim et al. 2005; Smith et al. 2004; Smith et al. 2006). However, the involvement and interaction of one or more of these transcription factors in regulation of FMR1 gene expression remains elusive. The frequency of occurrence of FXS is two-fold higher in males than in females and the pre-mutated male patients are known to show delay in brain development and suffer more from age-dependent neurodegenerative diseases. Moreover, the pattern of expression of FMR1 gene and the transcriptional regulatory mechanism of FMR1 gene during the development and aging processes especially in the male has not been well studied. Thus, the *Fmr1* gene expression and its regulation during life span in the normal brain in males are crucial for understanding the disease pathophysiology.

Therefore, in the present study, we have analysed the expression of *Fmr-1* gene at protein and transcript levels by Western blotting and RT- PCR as a function of age in the male mouse. Efforts also have been made to study whether alteration in the expression of *Fmr-1* gene is influenced by the interactions between Sp1 and USF1/USF2 to its cognate *Fmr-1* promoter and were studied during postnatal development and aging using electrophoretic mobility shift assay (EMSA) technique. The present communication also tries to establish a correlation between the expression of *Fmr-1* gene and the interaction of transcription factors Sp-1 and USF1/USF2 (E-box) to *Fmr-1* promoter in an age-dependent manner.

Materials and methods

Animals

AKR strain male mice (*Mus musculus*) were used for experimentation. Mice colony was maintained at 24±2^{o}C with 12-hours light and dark schedule and continuous access to food and drinking water ad libitum. The mice used for the experiments were 0 day old (newly born), 7 days old (Early postnatal age), 15 days old

(Middle-postnatal age), 45 days old (young), adult (20±2 weeks old) and old (70±5 weeks old). Mice were euthanized by IP injection of sodium pentobarbital (100mg/kg BW), brain was carefully removed and cerebral cortex was dissected out on ice. All experiments were performed following the guidelines of Institutional Ethical Committee of Banaras Hindu University for the use of experimental animals approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Govt. of India.

Chemicals

Analytical and molecular biology grade chemicals (Sigma & Merck) were used for all the experiments. Isotope [α-^{32}P] dCTP (specific activity 5000 Ci/mmol) was purchased from Board of Radiation and Isotope Technology, CCMB, Hyderabad (India). All the chemicals and enzymes were used as per manufacturer's instructions.

Tissue lysate preparation and Western blot analysis

The cerebral cortex was homogenized in 10X lysis buffer (50 mM Tris-Cl, pH 7.4) containing 0.2 % triton X-100, 5mM EDTA, 5mM EGTA, 2 mM PMSF, 5 mM benzamidine, 2 mM βME and protease inhibitor cocktail (Sigma-Aldrich) at 4^{o}C and centrifuged at 2,000X g for 30 min at 4^{o}C. The resulting supernatant containing total proteins was collected and the protein content was estimated by Bradford method (Bradford 1976). 50 µg of total protein was electrophoresed on 7.5% SDS polyacrylamide gel according to Laemmli (Laemmli 1970) and transferred onto the PVDF membrane (Millipore). The blot was incubated with mouse monoclonal anti-FMRP (1:2,000 dilution, Sigma, USA) or rabbit monoclonal anti-β-actin (1:25,000, Sigma-Aldrich, USA) in 5% non-fat milk in phosphate buffered saline (pH 7.4) overnight at 4°C. The blot was further treated with secondary antibody against mouse IgG conjugated with horseradish peroxidase (1:2,500) for 2 h at room temperature. FMRP and β-actin (internal control) signals were detected by an enhanced chemiluminescence (ECL) method and resulting signals on the X-ray film were densitometrically scanned separately. Scan data of FMRP was normalized with that of the β-actin to obtain relative density value (RDV) for FMRP.

Isolation of total RNA

Total RNA from cerebral cortex was isolated using TRI reagent (Sigma, USA) following User's manual. The aqueous phase collected was mixed with the equal volume (v/v) of isopropanol and precipitated at –70°C. The RNA pellet was collected, washed with ice-chilled 70% ethanol and dissolved in DEPC- treated water (Singh et al. 2007). Extracted RNA was treated with DNase-I (DNAfree™, Ambion) according to the manufacturer's guidelines to remove any DNA contamination. RNA concentration was calculated by measuring the absorbance by a UV–vis spectrophotometer at 260 nm. The integrity of the RNA samples was verified by 1% formaldehyde agarose gel electrophoresis.

Reverse transcription

For reverse transcription of RNA, 2.0 µg of RNA was mixed with 200ng of random hexamer primer (Fermentas) in sterile water in the reaction volume of 11.0 µl. It was incubated at 70°C for 5 min and chilled on ice. Thereafter, 4 µl of 5X reaction buffer, 2 µl of 10 mM dNTP mix and 0.5 µl (20 U) of RNase inhibitor (Ribolock™, Fermentas) were added, and the volume was made up to 19 µl with Deionized water. Tubes were incubated for 5 min at 25°C, and 1 ul (200 U) of M-MuLV reverse transcriptase (RevertAid TM H Minus, Fermentas) was added. Tubes were then incubated for 10 min at 25°C and thereafter at 42°C for 1 h in a thermal cycler (Bio-Rad, MJ Mini cycler™, Research). The reaction was terminated by heating at 70°C for 10 min followed by chilling them on the ice. They were stored at −70°C or directly used for the PCR reaction.

PCR amplification:

Expression of *Fmr-1*and β-actin was assessed by polymerase chain reaction (PCR) using the following gene-specific primers: 5'-TTACAGAAATAGG GGGCACG-3'and 5'-TACGCTGTCTGGCTTTTCCT-3' for *Fmr-1*; 5'-ATCGTGG GCCGCTCTAGGCACC-3' and 5'-CTCTTTGATGTCACGCACGATTTC-3'for β-actin. Initially, annealing temperature, the number of cycles, and annealing conditions were optimized for each *Fmr-1* and β-actin (Gaur and Prasad 2014; Pandey et al. 2015). The PCR amplified products of the genes were resolved by 2% agarose gel electrophoresis. Image of the gel was captured and *Fmr-1* and β-actin bands were densitometrically scanned separately. Scan data of *Fmr-1* was normalized with that of the β-actin to obtain relative density value (RDV) for *Fmr-1*.

End labelling of the *cis*-acting promoter elements:

The Sp1 and USF1/USF2 (E-Box) binding sequences were, respectively, found out from the DNA database and the complimentary single stranded oligonucleotides for 20 nucleotide (GCGGAGCGGAGA**GGGCGGG**G) -108 bp to -88 bp of *Fmr-1* gene promoter containing Sp1 binding site and 14 nucleotide E-Box (GGGT**CACGTG**A CAT) -51 bp to -37 bp of *Fmr-1* gene promoter containing USF1/USF2 binding site were custom synthesized and annealed to produce double-stranded oligonucleotides in 1X TNE buffer (10 mM Tris, 100 mM NaCl, and 1 mM EDTA). The oligos were denatured in the water bath at 95°C for 15 min and allowed to cool gradually. It was allowed to stand at room temperature overnight, and the annealed oligos (ds-oligos) were checked on 15% non-denaturing polyacrylamide gel (Fig.1). The annealed ds-oligos were labeled by an end-filling method using Klenow fragment of DNA polymerase. The 5' overhang was filled in a reaction volume of 20µl having 100ng of ds-oligos, 2.0 µl of 10X reaction buffer, 2.5 µl of 2mM dNTP mix (without dCTP), 50µCi α^{32}P-dCTP and one unit of Klenow enzyme (exo-) for 15 min. at 30°C. The reaction was stopped by heating the tube at 70°C for 10 min. Labeled probes were separated from free nucleotides by Sephadex-G50 column (spun column)

chromatography and its radioactivity content was measured by Beckman LS-100 liquid scintillation counter, and stored at -70°C (Gupta and Prasad 2014).

Electrophoretic mobility shift assay (EMSA):

Nuclear extract from cerebral cortex was prepared according to Gaur and Prasad 2014. DNA–nuclear protein interaction experiment was studied using EMSA technique. Before carrying out specific DNA-protein interaction in experimental conditions, pilot experiments were carried out for finding the optimum nuclear protein concentration in reactions with increasing concentration of nuclear extract. The optimum nuclear protein content for EMSA reactions was found to be 20 µg and 10 µg for Sp1, E-box respectively (Fig. 2a, 3a). For specificity competition assay, the cold probes (unlabeled) in various concentrations (50X, 100X, and 500 fold) were added with nuclear extract 10 min before the addition of ^{32}P-labeled probe in the reaction mix (Fig. 2b, 3b). Further, specificity of the binding was also confirmed by (nonspecific competition) binding between nuclear extract and cold heterologous poly-dI/dC (data not shown). 10-20µg of nuclear protein, as established from a pilot experiment, was taken in the binding buffer (5mM HEPES, pH 7.9, 10% glycerol, 25mM KCl, 0.05mM EDTA, 0.125mM PMSF) with 1µg of poly-dI/dC and 0.1-0.5ng of ^{32}P-labeled ds-oligo (approximately 8000-10,000 cpm) per reaction, separately and the mixture was incubated at 25°C for 30 min. The samples were resolved on 5% non-denaturing polyacrylamide gel, thereafter exposed to the intensifying cassette. The signals due to radiolabeled DNA-protein complexes were captured in Phosphor-Imager (GE, Typhoon FLA 7000).

Data and statistical analysis

All the experiments were carried out using 7 mice/ group of each age and repeated three times (n=21/age group). RDV for FMRP and *Fmr-1* transcript levels and the IDV for EMSA signals as quantified by computer-assisted densitometry (AlphaEase FCTM software, Alpha Innotech Corporation, CA) were shown as histograms having mean ± SEM from three sets of experiments. Data were statistically analyzed by One-way ANOVA followed by Student-Newman-Keuls test (SNK) using Sigma Stat 3.5 software. $P<0.05$ was considered statistically significant.

Results

Semi-quantitative RT-PCR analysis of *Fmr1*-mRNA transcript

Semi-quantitative RT-PCR of *Fmr-1* gene in the cerebral cortex (Fig.4a) of male mice reveals that *Fmr-1* transcript level follows the similar pattern as protein expression. *Fmr-1* mRNA expression is highest in 0, 7 and 15 days as compared to that in young, adult and old ($p<0.05$). In young mice, it was significantly down-regulated as compared to 15 day mice ($p<0.05$). Thereafter, during aging the transcript level gradually declined in adult and old male mice ($p<0.05$).

Western blot analysis of FMRP during postnatal development and aging

Fig.4b shows the Western blot of FMRP and β-actin expression in male mice of different ages. The FMRP and its isoforms were detected in the molecular weight range of ~69 to 80 kDa. There were three isoforms of FMRP and histogram shows the sum of the intensity of all the three isoform bands of FMRP. The expression of FMRP was higher in postnatal ages than in young, adult and old male. FMRP expression is higher in 0 day and 7 day mice and was gradually down-regulated with advancing age ($p<0.001$). A significant decline ($p<0.05$) in the level of FMRP in old male in comparison to young and the adult male was observed.

EMSA of Sp1 interaction with *Fmr-1* promoter

As per our EMSA result, a single complex (C) was formed between Sp-1 and *Fmr-1* promoter sequence (Fig. 5a). In order to correlate whether the interaction of Sp1 and USF1/USF2 (E-Box) with their cognate *Fmr-1* promoter sequence are altered during maturation and aging and levels *Fmr-1* expression, the EMSA was carried out. Nuclear extracts from male mice cerebral cortex were incubated with Sp-1 oligo, only one complex was formed. The intensity of Sp-1 complex was significantly high in 0-day old mice as compared to any other ages ($p<0.05$) and it's down regulating with age. Nuclear extracts when incubated with Sp-1 oligo, 15 day and young showed no change in the intensity but there was a significant decrease in adult and old, lowest intensity in old male mice (Fig. 5a & b).

EMSA of USF1/USF2 interaction with *Fmr-1* promoter

Incubation of the labeled oligo of E-box (USF1 and USF2) with nuclear extract of the cerebral cortex of male mice of different ages resulted in the formation of two complexes (Fig. 6a), but they were analyzed together due to their inadequate resolution. Analysis of this DNA-protein complex with different ages shows that the level of this DNA-protein complex was found to be significantly low in 0 day old mice as compared to other ages and peak at old age ($p<0.05$) with a gradual increasing pattern. There was a significant increase in the level of DNA-protein complex in the adult age ($p<0.05$) and this increase was maintained in old aged mice.

Correlation between *Fmr-1* gene mRNA expression and binding of Sp1 and USF1/USF2 to *cis*-acting elements of *Fmr-1* promoter

The *Fmr-1* gene expression at transcript level was correlated with the interaction of Sp1 and USF1/USF2 (E-Box) with their cognate *Fmr-1* promoter sequence during the period of postnatal development and aging. We noted a significant positive correlation ($r=0.946$) with the binding of Sp1 to *cis*-acting elements to *Fmr-1* promoter. Semi-quantitative RT-PCR analysis of *Fmr-1* mRNA level showed negative correlation (Fig.7) between *Fmr-1* mRNA expression and USF1/USF2 intensities ($r = -0.927$), in the male cerebral cortex as a function of age.

Discussion

The present study has demonstrated that *Fmr-1* gene expression was prominently high during the period of postnatal development and appears to be down-regulated throughout adulthood and aging in male mouse brain. This study also reveals the *Fmr-1* gene transcription regulation and its correlation with the interaction of Sp1 and USF1/USF2 transcription factors with the *Fmr-1* promoter.

We found that FMRP and *Fmr-1* mRNA expression was prominently high during postnatal development stages, while it showed a remarkable down-regulation in old age. FMRP acts as a regulator of neuronal morphology and synaptic pruning throughout the period of postnatal development and during adolescence, the higher level of FMRP expression that occurs during postnatal development in cortex may be necessary for the maintenance of connectivity and synaptic plasticity. *Fmr-1* knockout mice have been reported to display abnormalities in the myelination of cerebellar axons as early as in the first postnatal week. This reflects the vital role of FMRP in oligodendrocyte precursor cells survival, maturation and proliferation during the postnatal development (Pacey et al. 2013). Several studies also suggest the role of FMRP in neural differentiation, migration, axonal elongation and synaptogenesis, although experimental evidence are lacking (Bryant and Yazdani 2016; Michaelsen-Preusse et al. 2016). FMRP *via* MAP1B regulation modulates microtubule dynamics in the growing neurites, especially in the developing growth cones which is essential for the development of neuronal network (Gonzalez-Billault et al. 2005; Goold and Gordon-Weeks 2001; Gordon-Weeks 2004). In *Fmr-1* knockout neurons, the elevated MAP1B expression leads to aberrant microtubule dynamics, which alters the structural synaptic development. Abnormalities in dendritic spine maturation and synapse formation in the *Fmr-1* knockout neurons have also been correlated to the increased MAP1B expression (Lu et al. 2004). Altered microtubule dynamics in *Fmr-1* knockout neurons also control the trafficking of neurotransmitter receptors on the synapses and thus could affect the neurotransmitter-dependent synaptic communication (Huber et al. 2002; Zhao et al. 2015).

Our study with aging mouse revealed that the *Fmr-1* gene expression level was down-regulated throughout adulthood and aging. Down-regulation of *Fmr-1* expression in cerebral cortex in old can be well correlated with its known functions in synaptic plasticity which gets reduced during aging (Burke and Barnes 2006). Our results are consistent with the earlier report of Singh et al., (2007) which also suggest decreased *Fmr-1* expression during aging (Singh et al. 2007). Apart from its role as a translational repressor, a translational activator role for FMRP in regulation of superoxide dismutase (Sod1) gene expression has been reported. The high level of FMRP in the brain of developing mice may be related to the higher expression of Sod1 that prevents oxidative stress and favors proper synaptic growth during development. The increased oxidative stress in old age has been linked to decreased learning and memory (Bechara et al. 2009). The decreased level of FMRP in old age may lead to reduced stabilization of PSD95 which in turn may cause decreased NMDAR and AMPAR expression on the

postsynaptic membrane. The over-expression of various genes related to activation of NMDAR and AMPAR lead to LTD like situation. This may further cause decline in the memory and learning abilities during old age (Westmark 2013). High expression of *Fmr-1* gene during postnatal developmental and at young age may be associated with achieving cognitive abilities, whereas its low expression in old age may be linked to cognitive deficiencies. Changes in *Fmr-1* expression may be associated with the development- and age-dependent alterations in NMDA and AMPAR-dependent synaptic plasticity and hence learning and memory (Bostrom et al. 2015; Martin et al. 2016).

Alterations in the cellular content of transcription factors can severely affect the expression of critical genes involved in normal development. Switching on/off the expression of several genes occurs at various stages of the lifespan, more significantly during embryogenesis, postnatal development and during puberty and senescence. Disparity in the level of several transcription factors plays an important role in the control of gene expression during brain development and aging. Two most important transcription factors involved in brain development and ageing are Sp1 and USF1/USF2 (E-Box). These are also present in the *Fmr-1* promoter region and play a critical role in *Fmr-1* transcription (Kumari et al. 2005; Kumari and Usdin 2001). Therefore, we speculated that the postnatal development and age-dependent variations in the level of *Fmr-1*transcript and FMRP may be a result of interaction of these or the other transcription factors to their corresponding *Fmr-1* promoter. In present study we tried to find out how *Fmr-1* expression is regulated at the transcriptional level, whether the interaction of transcription factors Sp1 and USF1/USF2 with *cis*-elements of *Fmr-1* promoter vary during postnatal development and aging and are they accordingly associated with altered the expression of *Fmr-1*. Our EMSA results showed that the intensity of Sp-1 complex is highest during the postnatal period compared to other age groups and it declines with age.

We noted an age-dependent positive correlation of *Fmr-1* gene expression with Sp1 interaction level to the *Fmr*-1 promoter. Our observations are in line with earlier report of Luo et al., (2012) which suggests that expression of IL3 gene was high during postnatal day 1–4 and the affinity of SP1 to IL3 promoter can modulate its expression during later life span (Luo et al. 2012). The DNA-binding efficiency of Sp1 is reduced with aging and this reduction is not only due to the altered expression of Sp1 (Ammendola et al. 1992). Posttranslational modifications such as phosphorylation, acetylation, sumoylation, ubiquitylation, and glycosylation probably influence the transcriptional activity and stability of Sp1 (Tan and Khachigian 2009). Sp1 has also been reported to regulate the expression of several AD-related proteins, including amyloid precursor protein (APP), tau and the beta site APP cleaving enzyme 1 (BACE1) (Adwan et al. 2011). Studies have shown the involvement of Sp1 in regulation of genes that have been implicated in schizophrenia such as reelin, GAD67, MAOA/B, NMDA-receptor subunits NR1 and NR2A/B,GABA A and DA receptors D1A and D2/3 (Ben-Shachar 2009). It has been shown that Sp1 plays an important role

in regulation of D2 dopamine receptor gene transcription and alterations in Sp1 function may result in down-regulation of D2 gene expression during Huntington Disease (Dunah et al. 2002).

There are numerous literatures that indicate that methylation of genes, random mutation of gene promoter elements with age (Jones et al. 2015) or interaction between transcription factors and DNA are important regulators of gene expression. Singh and Prasad (2008) studied methylation of the upstream sequences of *Fmr-1* promoter which includes CpG islands in the 5'- untranslated region (UTR) and found that it gradually increases with the advancing age (Singh and Prasad 2008). Methylation may inhibit *Fmr*-1 transcription by blocking transcription factor binding site. Sp1 acts as an activator for basal SYN1 gene expression and CpG methylation can temper its activity thereby affecting SYN1 expression (Paonessa et al. 2013). Sp1 is an extremely versatile transcription factor involved in the expression of *Fmr-1* gene either as a main activator or in combination with other transcription factors like USF (Kumari et al. 2005). USF is a helix-loop-helix (HLH) transcription factor and its isoforms (USF1 and USF2) bind to E box elements. Our study reveals that USF1/USF2 interaction with *Fmr-1* promoter increases with advancing age and exhibit an age-dependent negative correlation with the *Fmr-1* gene expression. Singh and Prasad (2008) demonstrated that USF1/USF/2 interaction with *Fmr-1* promoter is altered with aging (Prasad and Singh 2008). Other report suggested that USF1 also interacts with the E-box in mouse Agtrap promoter and decreases Agtrap gene expression (Matsuda et al. 2013). More importantly, evidence suggests that the USF–CRE composite regulatory region plays a critical role in mediating activity-dependent gene expression in neurons (Chen et al. 2003). Steiger et al., 2004 also found that USF represses endogenous GABA R1b gene expression in the hippocampal neurons (Steiger et al. 2004). Study on CpG methylation-deficient *Drosophila* cells demonstrated that Nrf-1 and Sp1 are strong activators of an unmethylated human *FMR1*-driven reporter, whereas USF1/2 and Max repress this activation (Smith et al. 2004).

Our study summarily suggests that the interaction of SP1 and USF1/USF2 with the *Fmr-1* promoter plays a vital role in regulating the expression of *Fmr-1* gene during postnatal development and aging. In this regard, the interaction of SP1 and USF may serve as an important constitutive brake to regulate *Fmr-1* gene expression under aging conditions which ultimately may lead to deficient learning, memory and cognitive in old age. Studies on interaction of other transcription factors are required to link the decreased interactions of Sp1 and increased interactions of USF1/USF2 to their corresponding promoter sequences and decrease in *Fmr-1* expression due to aging. We are also analysing the behavioural experiments in aging mouse model as a function of *Fmr-1* gene expression and establishing the relevance of the interactions reported in the present study.

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Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflicts of interest.

REFERENCES:

Adwan LI, Basha R, Abdelrahim M, Subaiea GM and Zawia NH (2011) Tolfenamic acid interrupts the de novo synthesis of the beta-amyloid precursor protein and lowers amyloid beta via a transcriptional pathway Current Alzheimer research 8:385-392

Ammendola R, Mesuraca M, Russo T and Cimino F (1992) Sp1 DNA binding efficiency is highly reduced in nuclear extracts from aged rat tissues J Biol Chem 267:17944-17948

Bechara EG et al. (2009) A novel function for fragile X mental retardation protein in translational activation PLoS Biol 7:e16 doi:10.1371/journal.pbio.1000016

Ben-Shachar D (2009) The interplay between mitochondrial complex I, dopamine and Sp1 in schizophrenia Journal of neural transmission (Vienna, Austria : 1996) 116:1383-1396 doi:10.1007/s00702-009-0319-5

Birch RC, Hocking DR and Trollor JN (2016) Prevalence and predictors of subjective memory complaints in adult male carriers of the FMR1 premutation The Clinical neuropsychologist:1-15 doi:10.1080/13854046.2016.1145905

Bostrom CA, Majaess NM, Morch K, White E, Eadie BD and Christie BR (2015) Rescue of NMDAR-dependent synaptic plasticity in Fmr1 knock-out mice Cereb Cortex 25:271-279 doi:10.1093/cercor/bht237

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding Analytical biochemistry 72:248-254

Broek JA et al. (2016) Synaptic vesicle dynamic changes in a model of fragile X Mol Autism 7:17 doi:10.1186/s13229-016-0080-1

Bryant CD and Yazdani N (2016) RNA-binding proteins, neural development and the addictions Genes Brain Behav 15:169-186 doi:10.1111/gbb.12273

Burke SN and Barnes CA (2006) Neural plasticity in the ageing brain Nat Rev Neurosci 7:30-40 doi:10.1038/nrn1809

Chen WG et al. (2003) Upstream stimulatory factors are mediators of Ca2+-responsive transcription in neurons J Neurosci 23:2572-2581

Coffee B, Keith K, Albizua I, Malone T, Mowrey J, Sherman SL and Warren ST (2009) Incidence of fragile X syndrome by newborn screening for methylated FMR1 DNA American journal of human genetics 85:503-514 doi:10.1016/j.ajhg.2009.09.007

Dunah AW et al. (2002) Sp1 and TAFII130 Transcriptional Activity Disrupted in Early Huntington's Disease Science 296:2238-2243 doi:10.1126/science.1072613

Fraint A, Vittal P, Szewka A, Bernard B, Berry-Kravis E and Hall DA (2014) New observations in the fragile X-associated tremor/ataxia syndrome (FXTAS) phenotype Front Genet 5:365 doi:10.3389/fgene.2014.00365

Gaur P and Prasad S (2014) Alterations in the Sp1 binding and Fmr-1 gene expression in the cortex of the brain during maturation and aging of mouse Mol Biol Rep 41:6855-6863 doi:10.1007/s11033-014-3571-1

Gonzalez-Billault C et al. (2005) A role of MAP1B in Reelin-dependent neuronal migration Cereb Cortex 15:1134-1145 doi:10.1093/cercor/bhh213

Goold RG and Gordon-Weeks PR (2001) Microtubule-associated protein 1B phosphorylation by glycogen synthase kinase 3beta is induced during PC12 cell differentiation J Cell Sci 114:4273-4284

Gordon-Weeks PR (2004) Microtubules and growth cone function Journal of neurobiology 58:70-83 doi:10.1002/neu.10266

Grigsby J (2016) The fragile X mental retardation 1 gene (FMR1): historical perspective, phenotypes, mechanism, pathology, and epidemiology The Clinical neuropsychologist:1-19 doi:10.1080/13854046.2016.1184652

Gupta RK and Prasad S (2014) Differential regulation of GLT-1/EAAT2 gene expression by NF-kappaB and N-myc in male mouse brain during postnatal development Neurochem Res 39:150-160 doi:10.1007/s11064-013-1200-3

Hagerman RJ et al. (2001) Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X Neurology 57:127-130

Harris SW et al. (2008) Autism profiles of males with fragile X syndrome American journal of mental retardation : AJMR 113:427-438 doi:10.1352/2008.113:427-438

He CX and Portera-Cailliau C (2013) The trouble with spines in fragile X syndrome: density, maturity and plasticity Neuroscience 251:120-128 doi:10.1016/ j.neuroscience. 2012.03.049

Huber KM, Gallagher SM, Warren ST and Bear MF (2002) Altered synaptic plasticity in a mouse model of fragile X mental retardation Proc Natl Acad Sci U S A 99:7746-7750 doi:10.1073/pnas.122205699

Jacquemont S et al. (2004) Penetrance of the fragile X-associated tremor/ataxia syndrome in a premutation carrier population JAMA 291:460-469

Jones MJ, Goodman SJ and Kobor MS (2015) DNA methylation and healthy human aging Aging cell 14:924-932 doi:10.1111/acel.12349

Kelleher RJ, 3rd, Govindarajan A, Jung HY, Kang H and Tonegawa S (2004) Translational control by MAPK signaling in long-term synaptic plasticity and memory Cell 116:467-479

Kumari D, Gabrielian A, Wheeler D and Usdin K (2005) The roles of Sp1, Sp3, USF1/USF2 and NRF-1 in the regulation and three-dimensional structure of the Fragile X mental retardation gene promoter Biochem J 386:297-303 doi:10.1042/BJ20041124

Kumari D and Usdin K (2001) Interaction of the transcription factors USF1, USF2, and alpha - Pal/Nrf-1 with the FMR1 promoter. Implications for Fragile X mental retardation syndrome J Biol Chem 276:4357-4364 doi:10.1074/jbc.M009629200

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4 Nature 227:680-685

Lim JH et al. (2005) AP-2alpha selectively regulates fragile X mental retardation-1 gene transcription during embryonic development Hum Mol Genet 14:2027-2034 doi:10.1093/hmg/ddi207

Lu R et al. (2004) The fragile X protein controls microtubule-associated protein 1B translation and microtubule stability in brain neuron development Proc Natl Acad Sci U S A 101:15201-15206 doi:10.1073/pnas.0404995101

Luo XJ et al. (2012) The interleukin 3 gene (IL3) contributes to human brain volume variation by regulating proliferation and survival of neural progenitors PLoS One 7:e50375 doi:10.1371/journal.pone.0050375

Martin HG, Lassalle O, Brown JT and Manzoni OJ (2016) Age-Dependent Long-Term Potentiation Deficits in the Prefrontal Cortex of the Fmr1 Knockout Mouse Model of Fragile X Syndrome Cereb Cortex 26:2084-2092 doi:10.1093/cercor/bhv031

Matsuda M et al. (2013) Upstream stimulatory factors 1 and 2 mediate the transcription of angiotensin II binding and inhibitory protein J Biol Chem 288:19238-19249 doi:10.1074/jbc.M113.451054

Michaelsen-Preusse K, Zessin S, Grigoryan G, Scharkowski F, Feuge J, Remus A and Korte M (2016) Neuronal profilins in health and disease: Relevance for spine plasticity and Fragile X syndrome Proc Natl Acad Sci U S A 113:3365-3370 doi:10.1073/pnas.1516697113

Napoli E et al. (2011) Altered zinc transport disrupts mitochondrial protein processing/import in fragile X-associated tremor/ataxia syndrome Hum Mol Genet 20:3079-3092 doi:10.1093/hmg/ddr211

Niere F, Wilkerson JR and Huber KM (2012) Evidence for a fragile X mental retardation protein-mediated translational switch in metabotropic glutamate receptor-triggered Arc translation and long-term depression The Journal of neuroscience : the official journal of the Society for Neuroscience 32:5924-5936

Pacey LK et al. (2013) Delayed myelination in a mouse model of fragile X syndrome Hum Mol Genet 22:3920-3930 doi:10.1093/hmg/ddt246

Pandey SP, Rai R, Gaur P and Prasad S (2015) Development- and age-related alterations in the expression of AMPA receptor subunit GluR2 and its trafficking proteins in the hippocampus of male mouse brain Biogerontology 16:317-328 doi:10.1007/s10522-014-9548-6

Paonessa F, Latifi S, Scarongella H, Cesca F and Benfenati F (2013) Specificity protein 1 (Sp1)-dependent activation of the synapsin I gene (SYN1) is modulated by RE1-silencing transcription factor (REST) and 5'-cytosine-phosphoguanine (CpG) methylation J Biol Chem 288:3227-3239 doi:10.1074/jbc.M112.399782

Prasad S and Singh K (2008) Interaction of USF1/USF2 and alpha-Pal/Nrf1 to Fmr-1 promoter increases in mouse brain during aging Biochem Biophys Res Commun 376:347-351 doi:10.1016/j.bbrc.2008.08.155

Santos AR, Kanellopoulos AK and Bagni C (2014) Learning and behavioral deficits associated with the absence of the fragile X mental retardation protein: what a fly and mouse model can teach us Learn Mem 21:543-555

Schneider A, Ligsay A and Hagerman RJ (2013) Fragile X syndrome: an aging perspective Dev Disabil Res Rev 18:68-74 doi:10.1002/ddrr.1129

Singh K, Gaur P and Prasad S (2007) Fragile x mental retardation (Fmr-1) gene expression is down regulated in brain of mice during aging Mol Biol Rep 34:173-181 doi:10.1007/s11033-006-9032-8

Singh K and Prasad S (2008) Age- and sex-related analysis of methylation of 5'-upstream sequences of Fmr-1 gene in mouse brain and modulation by sex steroid hormones Biogerontology 9:455-465 doi:10.1007/s10522-008-9178-y

Smith KT, Coffee B and Reines D (2004) Occupancy and synergistic activation of the FMR1 promoter by Nrf-1 and Sp1 in vivo Hum Mol Genet 13:1611-1621 doi:10.1093/hmg/ddh172

Smith KT, Nicholls RD and Reines D (2006) The gene encoding the fragile X RNA-binding protein is controlled by nuclear respiratory factor 2 and the CREB family of transcription factors Nucleic Acids Res 34:1205-1215 doi:10.1093/nar/gkj521

Squire LR and Wixted JT (2011) The cognitive neuroscience of human memory since H.M Annual review of neuroscience 34:259-288

Steiger JL, Bandyopadhyay S, Farb DH and Russek SJ (2004) cAMP response element-binding protein, activating transcription factor-4, and upstream stimulatory factor differentially control hippocampal GABABR1a and GABABR1b subunit gene expression through alternative promoters J Neurosci 24:6115-6126 doi:10.1523/jneurosci.1200-04.2004

Tan NY and Khachigian LM (2009) Sp1 phosphorylation and its regulation of gene transcription Mol Cell Biol 29:2483-2488 doi:10.1128/mcb.01828-08

Till SM (2010) The developmental roles of FMRP Biochem Soc Trans 38:507-510 doi:10.1042/BST0380507

Waung MW and Huber KM (2009) Protein translation in synaptic plasticity: mGluR-LTD, Fragile X Curr Opin Neurobiol 19:319-326 doi:10.1016/j.conb.2009.03.011

Westmark CJ (2013) FMRP: a triple threat to PSD-95 Front Cell Neurosci 7:57 doi:10.3389/fncel.2013.00057

Zhao W, Wang J, Song S, Li F and Yuan F (2015) Reduction of alpha1GABAA receptor mediated by tyrosine kinase C (PKC) phosphorylation in a mouse model of fragile X syndrome International journal of clinical and experimental medicine 8:13219-13226

Legend to figures:

Fig.1 Polyacrylamide gel electrophoresis of annealed ds oligos. **ds**, double strand oligo; **ss**, single strand oligo.

Fig.2 (a) Optimization of nuclear extract concentration for Sp1 probe binding assay, (b) EMSA of DNA fragment containing Sp1 of *Fmr-1* promoter sequence with specific cold competitor. DNA–protein complexes were resolved on 5% native polyacrylamide gel. 20μg of NE was incubated with ^{32}P-labeled dsDNA of 20 - mer Sp1(-108). F- Free DNA; C- complex.

Fig.3 (a) Optimization of nuclear extract concentration for USF1/ USF2 probe binding assay, (b) EMSA of DNA fragment containing USF1/ USF2 of *Fmr-1* promoter sequence with specific cold competitor. DNA–protein complexes were resolved on 5% native polyacrylamide gel. 10μg of NE was incubated with ^{32}P-labeled dsDNA of 14 - mer USF1/ USF2 (-51). F- Free DNA; C- complex.

Fig.4 RT-PCR (a) and Western blot (b) analysis of *Fmr-1* gene expression in cerebral cortex of male mice of postnatal and aging. M- maker (100bp ladder). Histograms represent data expressed as mean ± S.E.M. from three different sets of experiments. * Significant from pervious age group; $P<0.05$. d- Day, Y-Young (45d), A-Adult (20w), O- Old (70w).

Fig.5 (a) EMSA of ^{32}P-labeled Sp1 oligo with nuclear extract showing DNA-protein binding in the cerebral cortex of male mice during postnatal development and aging. (b) Histogram represents the intensity of each complex as integrated density value (G). F- Free probe; C-complex *$P<0.05$, significant from previous age group. d- Day, Y-Young (45d), A-Adult (20w), O- Old (70w).

Fig.6. (a) EMSA of ^{32}P-labeled USF1/USF2 probe with nuclear extract showing DNA-protein binding in the male cerebral cortex during postnatal development and aging. (b) Histogram represents the intensity of each complex as integrated density value (G). . * Significant from pervious age group; # Significant from 0Day group, $ Significant from all age group $P<0.05$. d- Day, Y-Young (45d), A-Adult (20w), O- Old (70w).

Fig.7. Correlation pattern between *Fmr-1* mRNA expression and binding of (a) Sp1, (b) USF1/USF2 *cis*-acting elements of *Fmr-1* promoter in the male cerebral cortex during postnatal development and aging.

Figure: 1

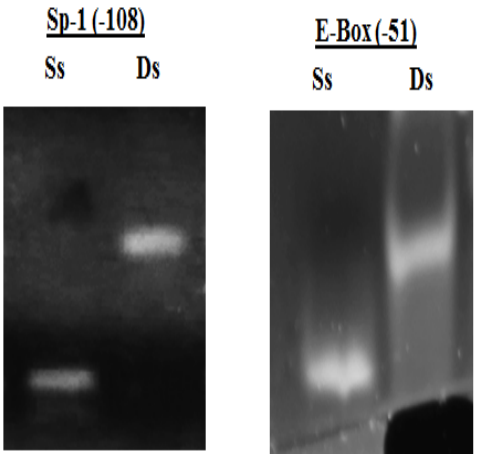


Figure: 2

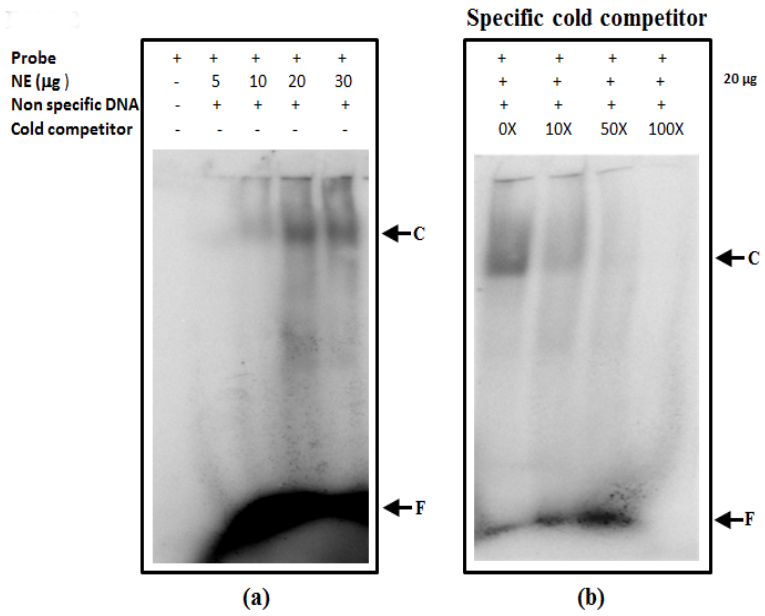
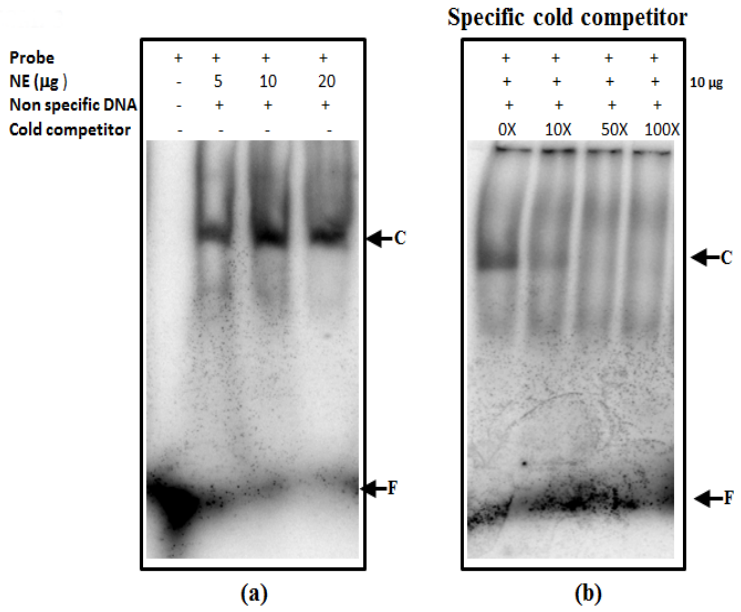


Figure: 3



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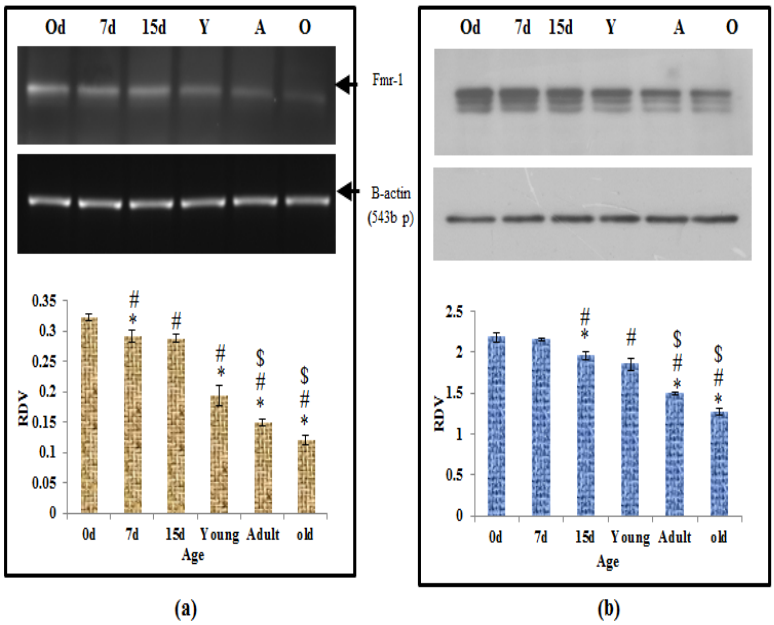

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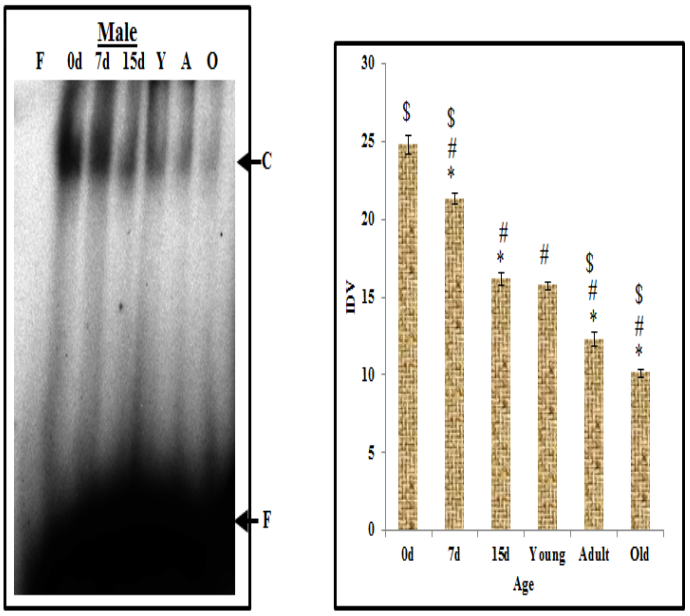

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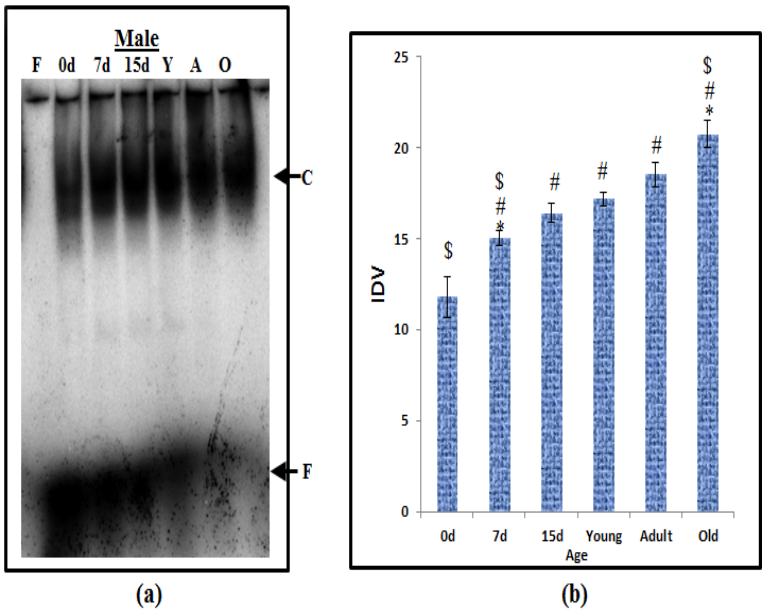

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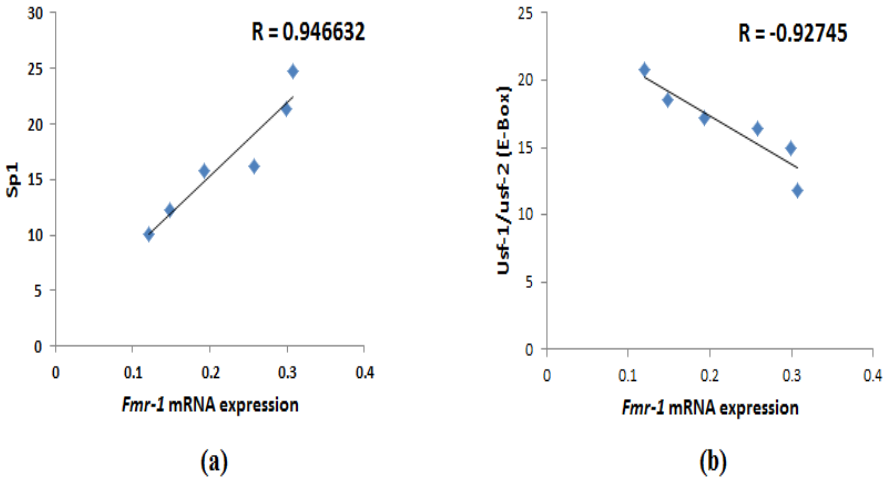
R = 0.946632
Sp1
Fmr-1 mRNA expression
(a)
R = -0.92745
Usf-1/usf-2 (E-Box)
Fmr-1 mRNA expression
(b)