Previous studies have demonstrated pathogenic outcomes upon expression of RNA containing expanded CGG repeats in various *in vitro/vivo* systems[1]–[7]. The pathogenicity induced by toxic RNA gain of function is mainly due to sequestration of specific RNA binding protein [8]–[11]. However, molecular mechanism of FMR1 mRNA containing expanded CGG repeats is unclear. In addition to RNA gain of function, RAN translation of expanded CGG repeats into toxic protein FMRpolyG may also contribute to FXTAS pathology. According to this CGG repeat expansion at 5'UTR of *FMR1* triggers RAN translation of FMR1 mRNA by non-canonical codon and produce poly glycine containing protein named as FMRpolyG. FMRpolyG has also been reported to be pathogenic and forming intranuclear inclusion in fly and human cell models [12]–[14]. Recent study using mice model also suggest the pathogenicity of expanded CGG repeats translated into FMRpolyG [15]. Hence, cytotoxicity induced by FMRpolyG was confirmed by expressing CGG repeats of premutation length in multiple cell lines. The findings here are in consonance with earlier reports in which RAN translation of an expanded CGG repeats leads to FXTAS pathogenesis.

5.1 Validation of plasmid constructs used to mimic *in vitro* FXTAS condition

The pathogenesis of FXTAS is mediated by expansion of 55-200 CGG triplets at the 5'-UTR of *FMR1* gene. Hence, to understand *in vitro* pathological mechanism of expanded CGG repeats we have used mainly two plasmid constructs named ATG FMRpolyG-GFP and 5'UTR *FMR1* CGG99X (gifted by Dr. Berguerand NC, [15]). Both the plasmids have 99XCGG repeats at 5'-UTR of FMR1 chimera sequence tagged with GFP and expressed under CMV promoter. Both plasmids only differ in presence of ATG/ACG at 5'-UTR before CGG repeats. ATG FMRpolyG-GFP contain canonical start codon 'ATG' which constitutively express FMRpolyG upon transfection (Fig 5.1A). While 5'UTR *FMR1* CGG99X contains non canonical codon 'ACG' which gets expressed by RAN translation, and more likely resemblesthe premutation condition (Fig. 5.1B). Restriction enzymes HindIII and XhoI were used to digest both the plasmids. HindIII and XhoI were able to digest both the plasmids into two fragments of corresponding lengths 369 bpand 4315 bp (Fig. 5.1C).

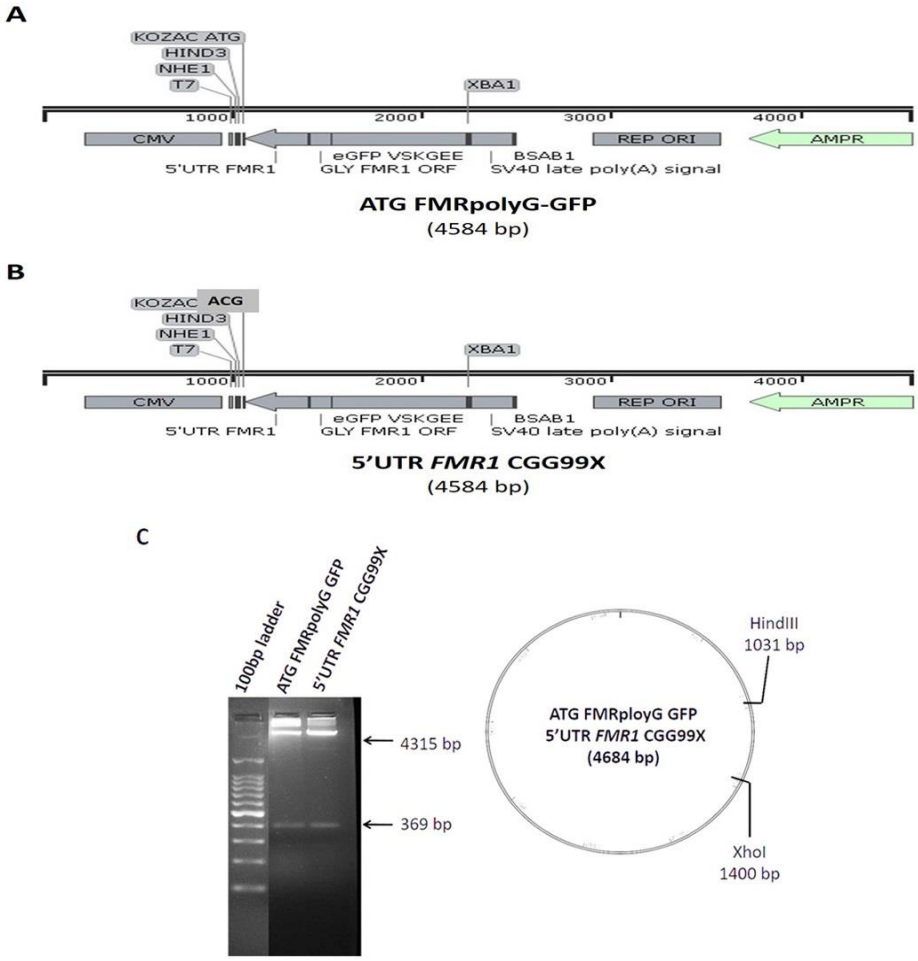

Figure5.1.Plasmids used to induce expression of 99XCGG repeats mimicking *in vitro*premutation condition.Linear representation of plasmid ATG FMRpolyG-GFP having canonical start codon 'ATG' at 5'UTR followed by 99XCGG repeats and FMRP chimera sequencetagged with GFP(A)and plasmid 5'UTR *FMR1* CGG99X having non-canonical start codon 'ACG' at 5'UTR followed by 99XCGG repeats and FMRP chimera sequencetagged with GFP (B).RE digestion of both the plasmid with HindIII and XhoI leads toformation of two fragments of expected lengths (shorter segment: 369 bp; longer fragment 4315 bp).

5.2 Expression of plasmid containing expanded CGG repeats decreases cellular viability.

Previous reports have suggested that expression of expanded CGG repeats embedded within the 5'UTR of *FMR1* shows cytotoxic effects in cell and animal models [6],[29],[30]. Hence, to evaluate the pathogenic effect of expanded CGG repeats, we transfected a plasmid expressing 99XCGG repeats embedded in the natural sequence of the human *FMR1* gene (Fig. 5.2A) into non-neuronal (HEK293) and neuronal (SH-SY5Y) cells. We first measured the release of lactate dehydrogenase(LDH) enzyme in the media which indicates damage in cell permeability. LDH

release assay showed impaired integrity of cell membranes upon expression of the CGG premutation plasmids compared to control in both HEK293 and SH-SY5Y transfected cells (Fig. 5.2B). The expression of expanded CGG repeats also reduces cellular metabolism as evidenced by a decreased production of the colored formazan product that originates from the NAD(P)H-dependent reduction of the MTT tetrazolium dye in in HEK293 and SH-SY5Y cells (Fig.5.2C). To assess whether this deleterious effect was due to the RAN translation of the CGG repeats into FMRpolyG, we assess the effect of expressing FMRpolyG-GFP cloned under the dependency of an artificial ATG start codon (Fig.5.2A). Transfection of FMRpolyG-GFP had also deleterious effect in SH-SY5Y cells (Fig.5.2C). Treatment of H_2O_2 (100 µM) for 4 hours used as positive control for ROS induced cell death [18]. Trypan blue exclusion assay was performed to check cell viability. Trypan blue staining showed the decreased viability in both CGG repeats transfected condition in HEK293 and SH-SY5Y cells (Fig5.2D)

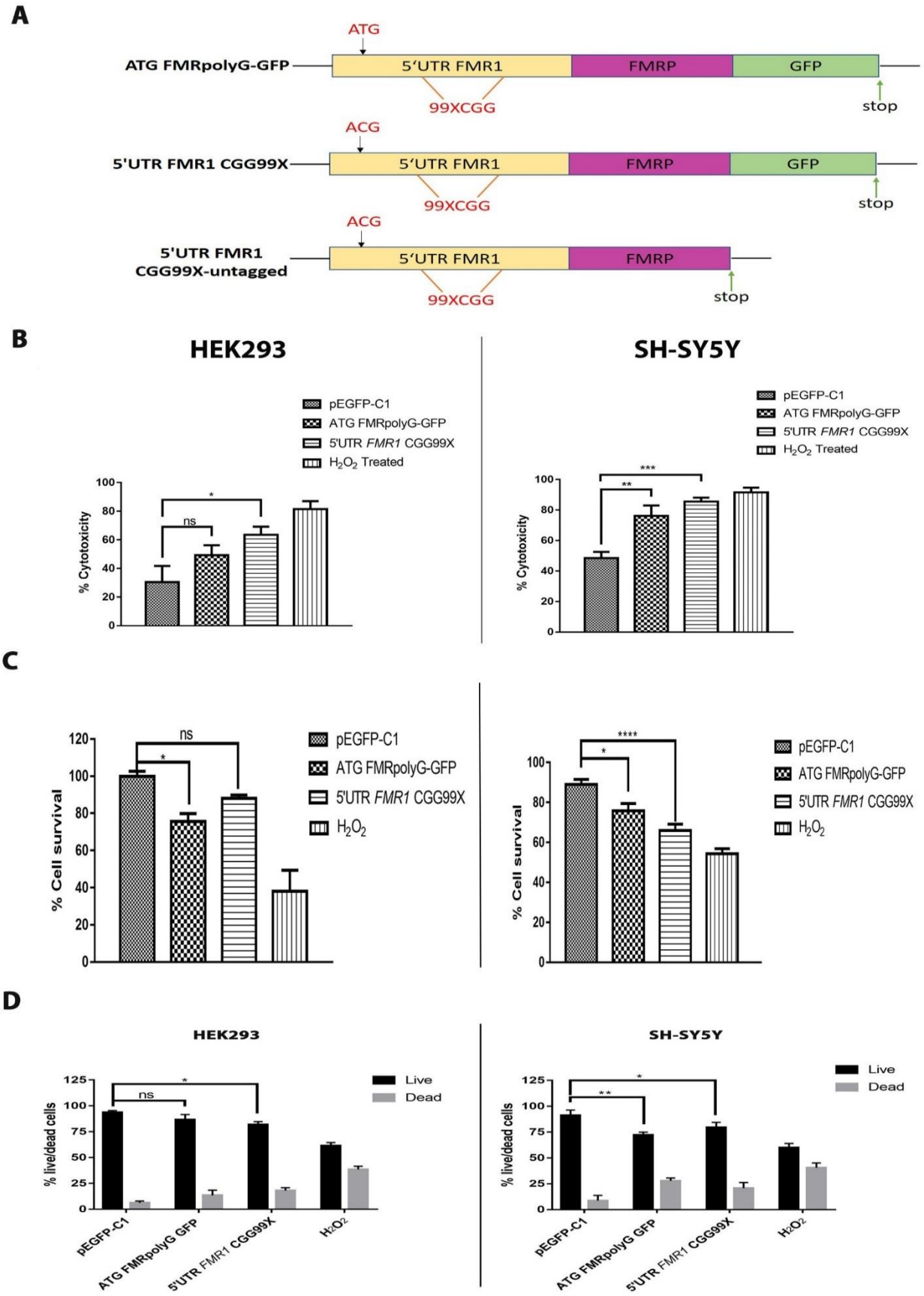
A
ATG
ATG FMRpolyG-GFP
5'UTR FMR1
FMRP
GFP
99XCGG
stop
ACG
5'UTR FMR1 CGG99X
5'UTR FMR1 CGG99X-untagged
B
HEK293
SH-SY5Y
pEGFP-C1
ATG FMRpolyG-GFP
5'UTR FMR1 CGG99X
H2O2 Treated
% Cytotoxicity
C
% Cell survival
H2O2
D
Live
Dead
% live/dead cells
pEGFP-C1
ATG FMRpolyG GFP
5'UTR FMR1 CGG99X
H2O2

Figure 5.2.Expression of plasmids containing expanded 99XCGG repeats affects cellular metabolism and causes decreased cell viability. Schematic representation of plasmids used to mimic*in vitro*geneticcondition of FXTAS in different cell lines (A). Both the constructs, ATG FMRpolyG-GFP and5'UTR*FMR1* CGG99X along with pEGFP-C1 (control) were transfected and abrupt cell permeability was measured by LDH release assay in HEK293 and SH-SY5y cells (B). Effect of FMR1 premutation on cellular metabolism checked by MTT assay (C) andTrypan blue staining assay was performed to count % live/dead cell population in HEK293 and SH-SY5y cells (D). (n=3) $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***)

We further explored CGG repeats induced altered mitochondrial metabolism by XTT assay. Transfection ofboth premutation plasmidsled to impaired mitochondrial metabolism as indicated by XTT tetrazolium reduction assays into HEK293 (human embryonic kidney), SH-SY5Y (human neuroblastoma) and U87MG (human glioblastoma) cells. (Fig.5.3A,B,C). Furthermore, propidium iodide staining also indicated decreased cell viability in the cells transfected with expanded CGG repeats as compared control (Fig.5.3D,E,F). Of interest, expression of ATG FMRpolyG-GFP, a construct with mutation of the natural non-canonical ACG start codon of FMRpolyG into an ATG canonical start codon enhanced FMRpolyG expression and results into reduced metabolism and increased cell death (Fig 5.3D,E,F).

UTR

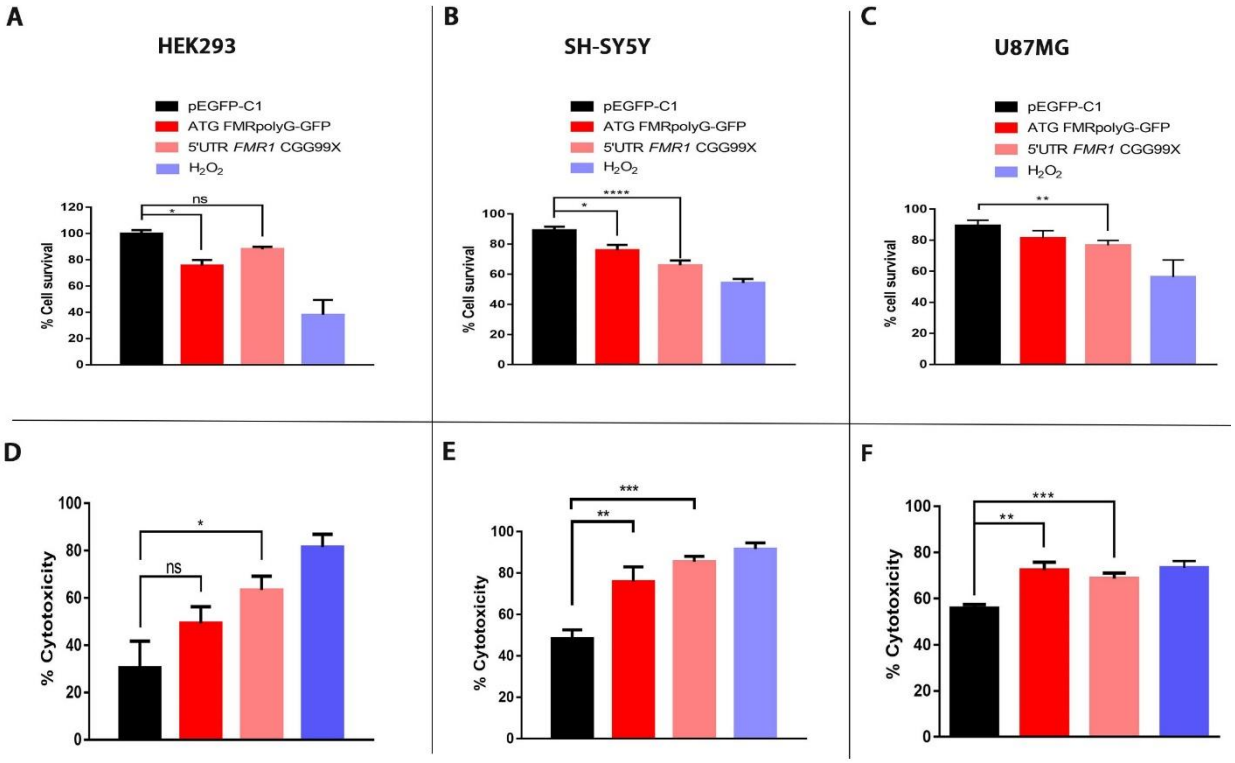

Figure 5.3.Expression of expanded CGG repeats causes decreased cell survival and increased apoptosis. Constructs expressing CGG repeats (ATG FMRpolyG-GFP and 5'UTR *FMR1* CGG99X)were transfected, and cellular viability wasdetermined by XTT assay in HEK293 (A), SH-SY5Y(B) and U87MG (C) cells. FMRpolyG inducedcytotoxicity in HEK293 (D), SH-SY5Y (E), and U87MG (F) cells was measured by staining the cells with Propidium Iodide (PI) (n=3) $p > 0.05$ (ns), $p \leq 0.05$ (*),$p \leq 0.01$ (**), $p \leq 0.001$ (***).

5.3 FMRpolyG induce caspase activation and increased cell death markers

The common signaling cascades involved in apoptosis is the activation of a highly specialized family of cysteinyl-aspartate proteases (caspases) which are usually present as inactive zymogen forms [19]. Hence, we firstanalysed the levels of caspase3/7 activity.The expression of either ATG FMRpolyG-GFPor FMRpolyG-GFP increases caspase 3/7 activity suggesting the activation of caspase dependent apoptosis in HEK-293 (Fig. 5.4A) and in SH-SY5Y (Fig. 5.4B) cells. The activation of initiator caspases such as caspase 8 and caspase 9 in both CGG repeats transfected condition were also analysed in HEK293 and SH-SY5Y cells.

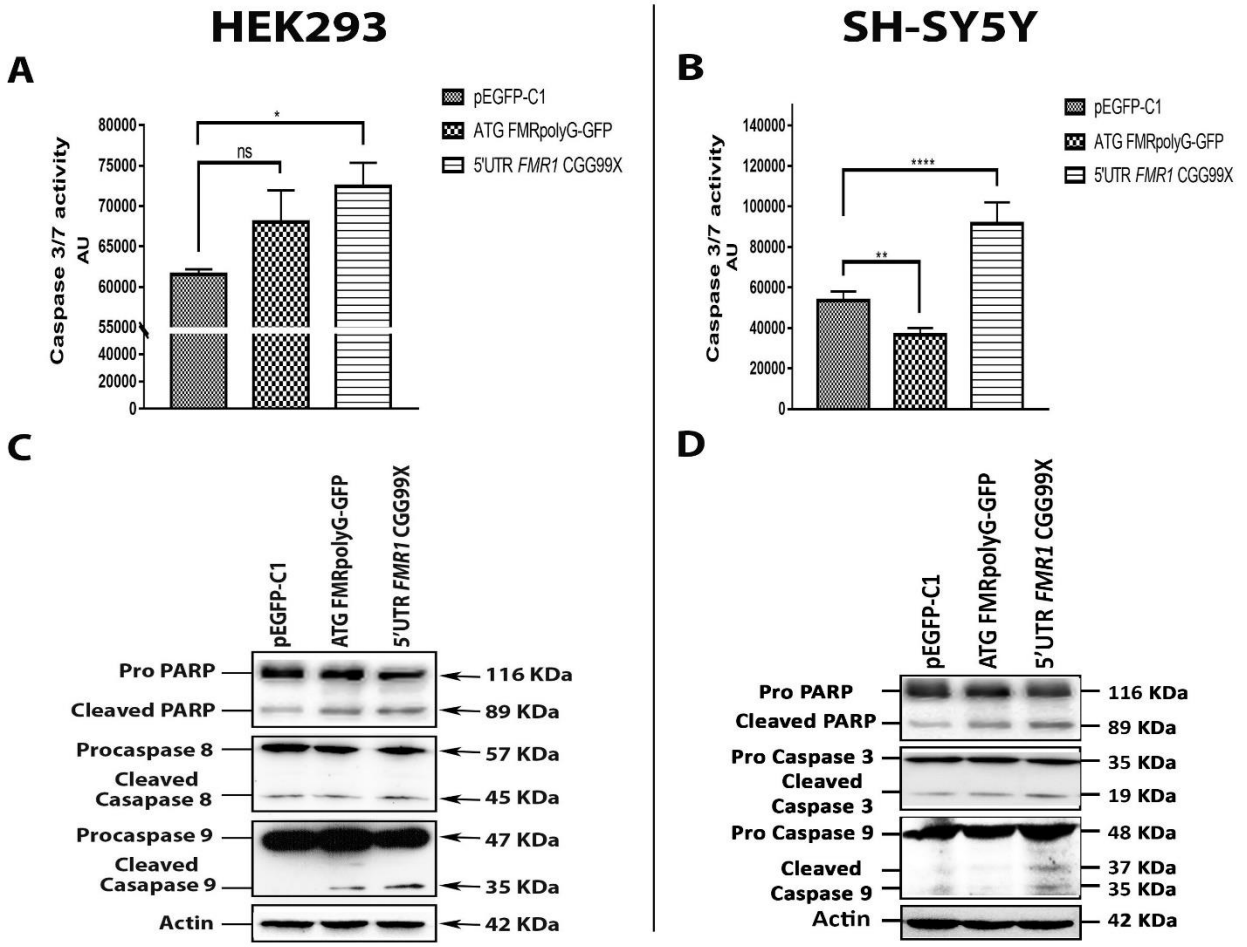

Figure5.4.Expression of expanded CGG repeats activates caspases and causes apoptotic cell death. Constructs for FMR premutation (ATG FMRpolyG-GFP and5'UTR*FMR1* CGG99X) along with pEGFP-C1 (control) were transfected and (A), and SH-SY5Y (B) cells. Levels of Caspase 3/7 activity was analysed in HEK293 (C) and SH-SY5Y (D) cells. Western blot analysis was performed for apoptotic markers such as caspase 8, caspase 9, caspase 3 and PARP in HEK293 (E), and SH-SY5Y (F). (n=3) $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***)

Immunoblotting results showed increased level of 35 KDa band corresponding to the cleaved subunit of caspase 9 under both CGG repeats expressed condition in HEK293 (Fig. 5.4C) and SH-SY5Y (Fig.5.4D) cells. There was no change in cleaved form of caspase 8 in CGG repeats transfected condition as compared to control. These results indicate involvement of mitochondrial mediated apoptosis. Caspase mediated apoptotic cell death is accomplished by the cleavage of several key proteins involving PARP (Poly ADP-ribose polymerase) [20]. Hence, we also checked the levels of pro/cleaved form of PARP. Increased levels of cleaved PARP also suggest

the induction of apoptosis in repeats transfected cells (Fig.5.4C,D). Overall, these results confirm that expression of CGG repeats promotes cellular toxicity and induces mitochondrial mediated apoptosis in cells derived from different origin.Overall, these results are consistent with previous reports [9],[14],[31] and confirm that expression of CGG repeats alters cellular metabolism and promotes cellular toxicity in cells from different origin.

6 Discussion

FXTAS is a single gene disorder caused by an expansion of 55-200 CGG repeats located within the 5'-UTR of *FMR1*. Previous studies have suggested that RAN translation of the CGG permutation into the FMRpolyG protein is an important cause of pathological changes in FXTAS [15], [21]. Corollary, our study also indicates an important role of FMRpolyG in premutation pathology and compliments the previous studies where expression of FMRpolyG was proved to be pathogenic in Drosophila and transgenic mice models [4], [12], [15]. We have used the plasmid 5'UTR *FMR1* CGG99X to understand the pathological consequences of RAN translation of expanded CGG repeats into FMRpolyG. While the other premutation construct ATG FMRpolyG-GFP was used as positive control as ittends to express FMRpolyG constitutively under canonical start codon ATG.

We performed the experiments in neuronal and non-neuronal cells, and expressed the constructs mimicking FXTAS condition to check the FMRpolyG induced cytotoxicity. The enforced expression of FMRpolyG either by ATG or RAN translation mimicking native FXTAS condition in different cell lines induedcytotoxicity and inhibited cell proliferation.However, our results are largely based on overexpression systems and do not exclude a potential pathogenic contribution of the CGG repeats at the RNA level. Thus, it remains to test whether only exogenous expression of FMRpolyG affects cellular viability or its combinatorial effect of RNA gain of function and FMRpolyG induced toxicity. Similarly, cytotoxic studies upon expression of constructs expressing only CGG repeats versus constructs expressing FMRpolyG with optimized codon to exclude CGG repeats toxicity will be necessary to conclude about the relative importance of CGG RNA toxicity versus CGG-RAN translation pathogenicity.