6. MITA acts as tumor suppressor in breast cancer

6.1. MITA positively regulate cell death in breast cancer

MITA is an antiviral signaling protein, which is responsible for inducing IFNs and NF-κB to mount innate immune response against infection [17]. Moreover, MITA is observed to be down regulated in breast cancer which suggests its role in regulation of cell survival. We hypothesize that MITA may act as tumor suppressor by regulating cell death in breast cancer. The expression of MITA was least in four different cell lines studied; hence further experiments were performed in MCF-7 cell line. MITA was expressed in MCF-7 to understand its role in regulation of cell survival. Cell proliferation was monitored using MTT in MITA transfected MCF-7 cells. There was a significant decrease observed in case of MITA transfected MCF-7 cells compared to vector control. The decreased reduction in MTT dye indicates decreased cell survival in the presence of MITA (Fig 6.1A). To further validate the role of MITA in cell survival, MITA was transfected and expressed in T47D cells and cell survival was monitored using MTT. Similar to MCF-7, decreased cell survival was observed in the presence of MITA in T47D cells (Fig 6.1B). This suggest that role of MITA is not cell line specific.

MITA induced decrease in cell survival further suggests that MITA may regulate cell death. The hypothesis was confirmed by monitoring cell death in the presence of MITA. MITA was transfected and expressed in MCF-7 and cell death was monitored using trypan blue exclusion assay. We observed that number of trypan blue positive cells increased in the presence of MITA as compared to vector transfected cells. The result indicates that expression of MITA induces cell death in MCF-7 cells (Fig-6.1C). MITA induced cell death was further confirmed using PARP cleavage assay. PARP is cleaved by caspases during apoptosis process and is the marker of apoptosis [123, 124]. MCF-7 cells were transfected with MITA and PARP cleavage was monitored using western blot analysis. Significant increase in the 89 KDa band representing cleaved PARP was observed in western blotting assay (Fig 6.1D).

6.2. Caspases play an essential role in MITA induced cell death

During the process of apoptosis, caspases cleaves and inactivates the essential DNA repair protein PARP as discussed above. The release of cleaved PARP clearly indicates the activation of caspases; hence caspase activation was monitored using caspase 3/7 assay.

MCF-7 cells were transfected with MITA and caspase 3/7 activity was monitored using luminometer. Caspase activity was observed to increase in the presence of MITA as compared to vector control (Fig 6.2A).

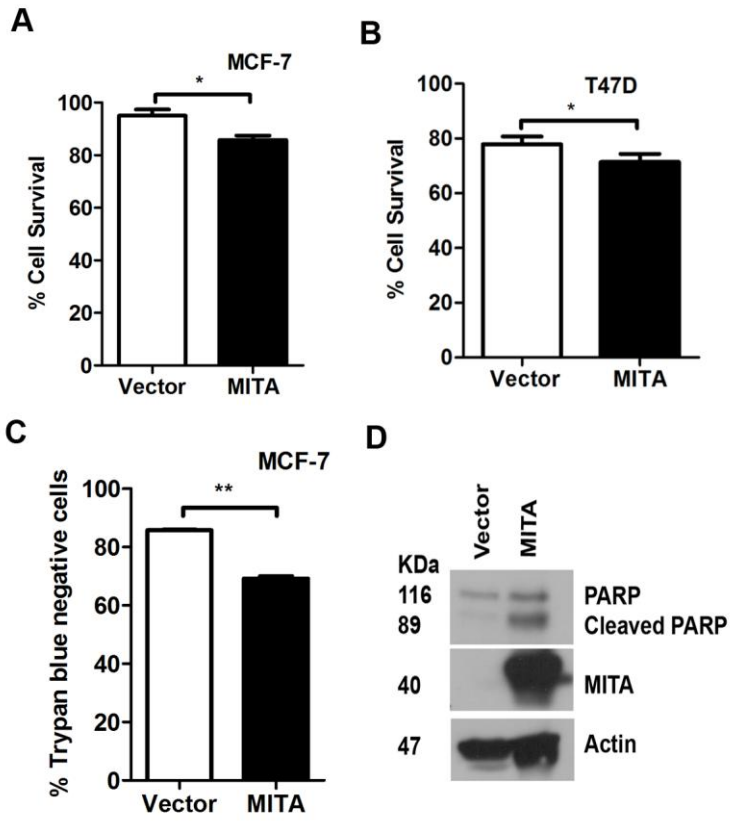

Figure-6.1: MITA induces cell death in breast cancer:

MITA was expressed in MCF-7 cells (A) as well as T47D cells (B) and cell survival was monitored using MTT assay. (C) MITA was transfected in MCF-7 cells and cell death was monitored using trypan blue exclusion assay and (D) PARP cleavage assay.

To avoid cell line specific response, caspase activity was monitored in T47D cell line. MITA was transfected and expressed in MCF-7 cells. Similar to MCF-7 the presence of MITA induced caspase activation in T47D cells (Fig 6.2B). As observed in figure 6.1, HBL100 showed highest expression of MITA; hence for further validation of the results, MITA was knocked down in HBL100 cells using MITA shRNA. HBL100 cells were cotransfected with FDEVDG2 (reporter construct) construct and MITA shRNA and caspase activity was monitored. Caspase activity decreased significantly in MITA

knockdown condition. The results clearly indicate that MITA induces cell death in breast cancer cells. The further question was to understand weather the

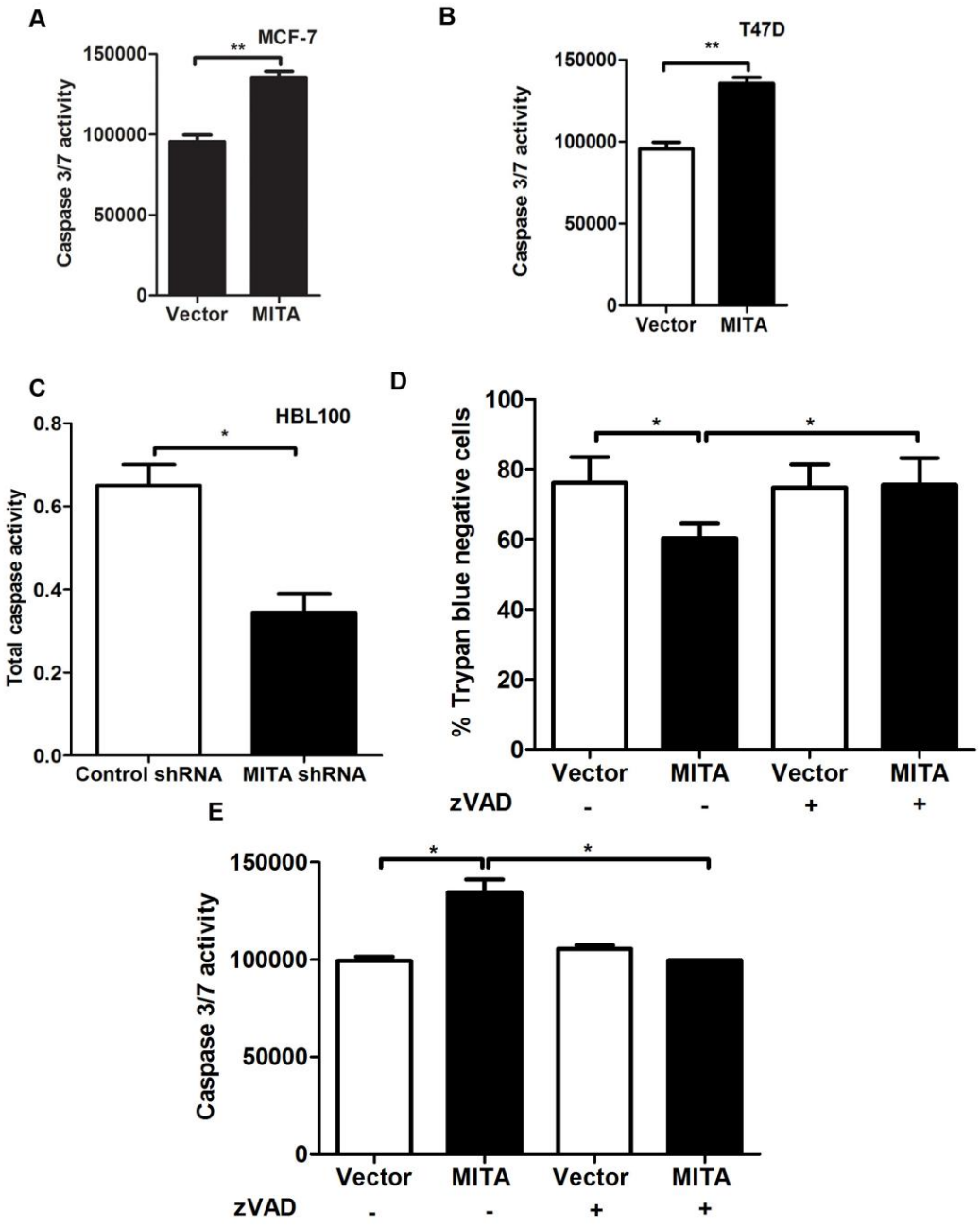

Figure-6.2: Caspases play an essential role in MITA induced cell death:

MITA was expressed in MCF-7 cells (A) as well as T47D (B) and caspase activity of monitored using luciferase based Caspase 3/7 assay. (C) MITA was knocked down in HBL100 cells and caspase activity monitored. (D) MITA transfected MCF-7 cells were

treated with zVAD-fmk (20μM) for 24 hrs and cell death as well as (E) caspase activity was measured using trypan blue exclusion assay and caspase 3/7 assay respectively.

activated caspases aids in inducing apoptosis; hence cell death was monitored in the presence of PAN caspase inhibitor zVAD-fmk using trypan blue exclusion assay. If caspases are essential for cell death, inhibition of caspases will rescue from MITA induced cell death. Results indicated that zVAD-fmk significantly increased trypan blue negative cells as compared to control and thus rescued from MITA induced cell death (Fig 6.2C). The inhibition of caspases was also confirmed using caspase 3/7 luciferase assay. The caspase activity decreased significantly in the presence of zVAD-fmk (Fig 6.2D). It is thus clear that the rescue from MITA induced cell death is due to the inhibition of caspases.

6.3. MITA leads to NF-κB activation

During antiviral signaling, MITA is known to up regulate NF-κB, a regulator of pro and anti apoptotic genes [125, 126]. We hence hypothesize that NF-κB might play an essential role in MITA mediated cell death. MCF-7 cells were cotransfected with MITA and NF-κB luciferase reporter construct to assess NF-κB activation. MITA expression led to significant increase in luminescence and thus the activation of NF-κB (Fig 6.3A). The activated form of NF-κB is a heterodimer consisting of a p50 subunit and p65, and the expression of p65 is positively regulated by NF-κB [127]. The result was further confirmed using western blot analysis in MITA transfected cells. MITA was expressed in MCF-7 cells and p65 levels were detected using western blot analysis (Fig 6.3B). The results clearly showed significant increase in the 65 KDa band corresponding to p65[128]. p65 is known to translocate to nucleus upon NF-κB activation; hence p65 levels were also monitored in nuclear fraction of MITA transfected cells. MITA was transfected in MCF-7 cells, treated with TNFα and nuclear fraction was isolated. TNFα was taken as a positive control as it is known to induce nuclear translocation of NF-κB [128]. p65 levels increased significantly in the nuclear fraction in the presence of MITA (Fig 6.3C). The nuclear fractionation analysis suggested the nuclear translocation of p65.

The NF-κB activation is mainly regulated by IKK kinase complex (IKKα, IKKβ and IKKγ). These kinases form a complex and phosphorylate inhibitor of NF-κB (IκBα) [127].

This leads to the release and nuclear translocation of NF-κB. The components of kinase complex were knocked down using shRNAs against IKKα, IKKβ and IKKγ to further understand the mechanism of MITA induced upregulation of NF-κB. MCF-7 cells were cotransfected with MITA and specific shRNAs and NF-κB activation was monitored using NF-κB luciferase assay. It was observed that IKK knockdown inhibited MITA induced NF-κB activation. This suggested that MITA mediated NF-κB activation requires IKK complex activity. The experimental evidences strongly suggest that MITA acts at IKK complex and activates NF-κB (Fig 6.3D).

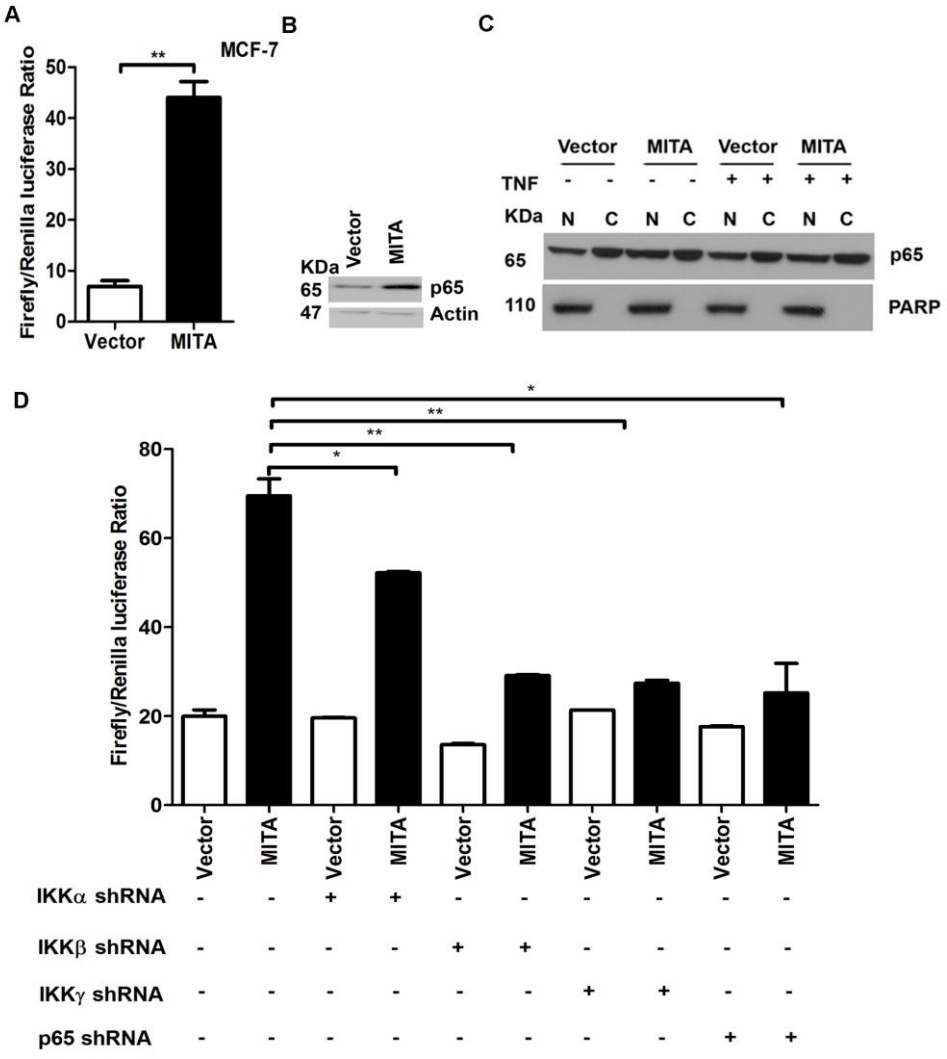

Figure-6.3: MITA activates NF-κB:

(A) MITA was expressed in MCF-7 cells and NF-κB activation was monitored using NF-κB luciferase assay. (B) MITA was transfected in MCF-7 cells. After 24 hrs of transfection cells were collected and lysed. Western blot analysis of the lysate was performed and levels of p65 were monitored using antibody specific to p65. (C) MITA was transfected in MCF-7 cells. After 24 hrs of transfection cells were collected and nuclear fraction was isolated. Western blot analysis of the isolated fraction was performed and levels of p65 were monitored using antibody specific to p65 in the nuclear fraction. (D) MCF-7 cells were cotransfected with MITA and shRNA specific to IKK complex components. After 24 hrs of transfection, NF-κB levels were monitored using NF-κB luciferase assay.

6.4. NF-κB regulates MITA induced cell death

MITA induces cell death as well as leads to NF-κB activation as evident from the above observations. NF-κB is an essential regulator of pro and anti apoptotic genes; hence we further questioned whether MITA mediated NF-κB is essential for MITA mediated cell death. To understand the role of NF-κB in MITA mediated cell death, p65 was knocked down in MITA transfected MCF-7 cells and cell death was monitored using trypan blue exclusion assay. p65 knockdown significantly decreased trypan blue positive cells in MITA transfected MCF-7 cells (Fig 6.4A). This indicates that p65 knockdown rescues from MITA induced cell death. The same was confirmed using chemical inhibitor of NF-κB. MITA transfected MCF-7 cells were treated with PDTC, a chemical inhibitor of NF-κB and cell death was monitored using trypan blue exclusion assay. PDTC treatment significantly decreased trypan blue positive MITA transfected cells as observed in p65 knockdown condition (Fig 6.4B). The results clearly indicates that MITA mediated cell death is regulated by NF-κB in MCF-7 cells.

Results indicated that caspases as well as NF-κB activation, both are essential for MITA induced cell death. We henceforth checked the role of NF-κB activation on caspase activation. p65 shRNA and MITA were cotransfected in MCF-7 cells and caspase activity was monitored. p65 knockdown significantly decreased the caspase activity in MITA transfected cells further confirming the role of NF-κB in MITA induced cell death (Fig 6.4C). Similarly, PDTC treatment to MITA transfected cells also inhibited caspase

activation in MITA transfected cells suggesting NF-κB inhibition rescue from MITA induced cell death (Fig 6.4D). Role of NF-κB in MITA induced cell death was further confirmed by PARP cleavage assay under p65 knockdown condition. p65 was knocked down in the MITA transfected MCF-7 cells and PARP cleavage was monitored using western blot analysis. The result showed significant decrease in the 89 KDa band corresponding to cleaved PARP in the p65 knockdown condition in MITA transfected cells as compared to control (Fig 6.4E). The results thus suggest that MITA activated NF-κB activation is essential for MITA induced cell death.

6.5. NF-κB regulates MITA induced cell death by upregulating TNFα

NF-κB activation leads to the induction of several genes regulating apoptosis[126]; hence for the better understanding of MITA mediated cell death NF-κB responsive genes were analyzed. MCF-7 cells were transfected with MITA and the expression of NF-κB responsive genes (BCL-XL, BCL-2, Bax, TNF-α, cIAP1, cIAP2 and XIAP) were analyzed using quantitative real time PCR. Out of the NF-κB responsive genes screened, BCL-XL, BCL-2, Bax, cIAP1, cIAP2 and XIAP showed little difference (Fig 6.5A) but TNFα expression was observed to be enhanced several fold (Fig 6.5B). Role of TNFα in MITA induced cell death was further investigated. MITA transfected MCF-7 cells were treated with TNFα and cell death was monitored using trypan blue exclusion assay. Interestingly, TNFα treatment further sensitized MITA induced cell death as compared to control (Fig 6.5C). The result suggests that MITA activates NF-κB which leads to enhanced TNFα production that leads to cell death.

Capase-8 plays a major role in TNFα induced apoptosis[129]. The role of caspase-8 was hence analyzed in MITA induced cell death. MCF-7 cells were transfected with MITA and caspase-8 activation was analyzed using western blot analysis. The enhanced level of 43/41 kDa band corresponding to cleaved caspase-8 was clearly observed in MITA transfected cells as compared to control (Fig 6.5D). To further confirm the role of caspase-8 in MITA induced cell death, cell death was monitored in the presence of caspase-8 inhibitor z-IETD-fmk. MCF-7 cells were transfected with MITA and treated with z-IETD-fmk and cell death was monitored using trypan blue exclusion assay. There was a

significant decrease observed in trypan blue positive cells in IETD treated MITA transfected cells as compared

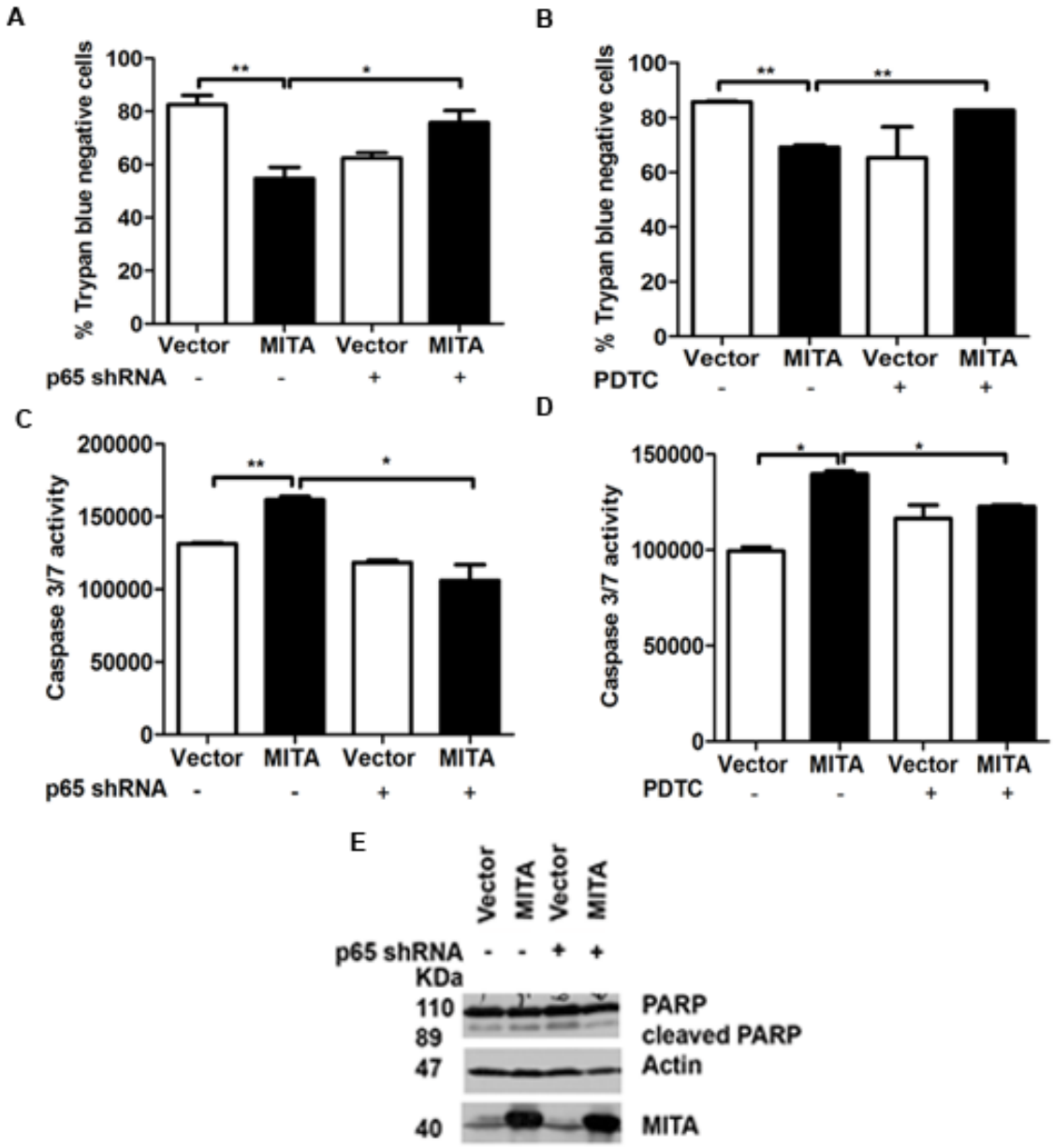

Figure-6.4: MITA activated NF-κB regulates cell death in MCF-7 cells

(A) MITA and p65 shRNA were cotransfected in MCF-7 cells and cell death was monitored using trypan blue exclusion assay. (B) MCF-7 cells were transfected with MITA and treated with PDTC (100μM) and cell death was monitored using trypan blue exclusion assay. (C) MITA and p65 shRNA were cotransfected in MCF-7 cells and

caspase activation was monitored using caspase 3/7 assay. (B) MCF-7 cells were transfected with MITA and treated with PDTC (100μM) and caspase activation was monitored using caspase 3/7 assay.

to control (Fig 6.5E). Role of caspase-8 in MITA induced cell death was further confirmed by knocking down MITA in HBL100 cells. MITA shRNA was transfected in HBL100 and caspase-8 activation was analyzed using luminescence based caspase-8 assay. MITA knockdown significantly decreased caspase-8 activity in HBL100 cells (Fig 6.5F). It was observed that NF-κB regulates MITA induced cell death; hence we further tasted role of NF-κB in caspase-8 activation. MCF-7 cells were cotransfected with p65 shRNA and MITA and cell death and caspase-8 activation was analyzed using luminescence based caspase-8 assay. p65 knockdown significantly inhibited MITA induced caspase-8 activation (Fig 6.5G). The results clearly indicate that caspase-8 plays a major role in MITA induced cell death.

6.6 MITA regulates tumorigenic potential of breast cancer cells

The results indicate that MITA is downregulated in breast cancer cells as well as breast cancer patients. Moreover, it also induces cell death in MCF-7 which is regulated by NF-κB. Therefore, we hypothesized that MITA may regulate tumorigenic potential of MITA atleast in breast cancer. The hypothesis was tasted by monitoring clonogenic ability of MCF-7 cells in the presence of MITA. MCF-7 cells were expressed with MITA and clonogenic ability was monitored using colony forming assay. MITA significantly decreased the no. of colony forming units of MCF-7 cells as compared to vector control (Fig6.6A). As NF-κB was observed to regulate MITA mediated cell death; role of p65 was further analyzed in clonogenic ability of MCF-7 cells. MCF-7cells were cotransfected with p65 shRNA and MITA and clonogenic ability of MCF-7 cells were monitored. p65 knockdown significantly increased the no. of colony forming units of MITA transfected cells as compared to control (Fig 6.6B).

The tumorigenic potential of MITA was further analyzed by analyzing the migration ability of breast cancer cells. MCF-7 cells were transfected with MITA and migration ability of the cells was analyzed using scratch assay. MITA expression led to increased open wound area as compared to control (Fig 6.6C). The result was further confirmed by

knocking down MITA in HBL100 cells. MITA shRNA was transfected in HBL100 cells and migration ability of HBL100 cells was monitored using scratch assay. MITA knockdown significantly decreased the area of open wound area as compared to control (Fig 6.6D). The results clearly suggest that MITA regulates tumorigenic potential of breast cancer cells. MITA thus acts as a tumor suppressor in breast cancer.

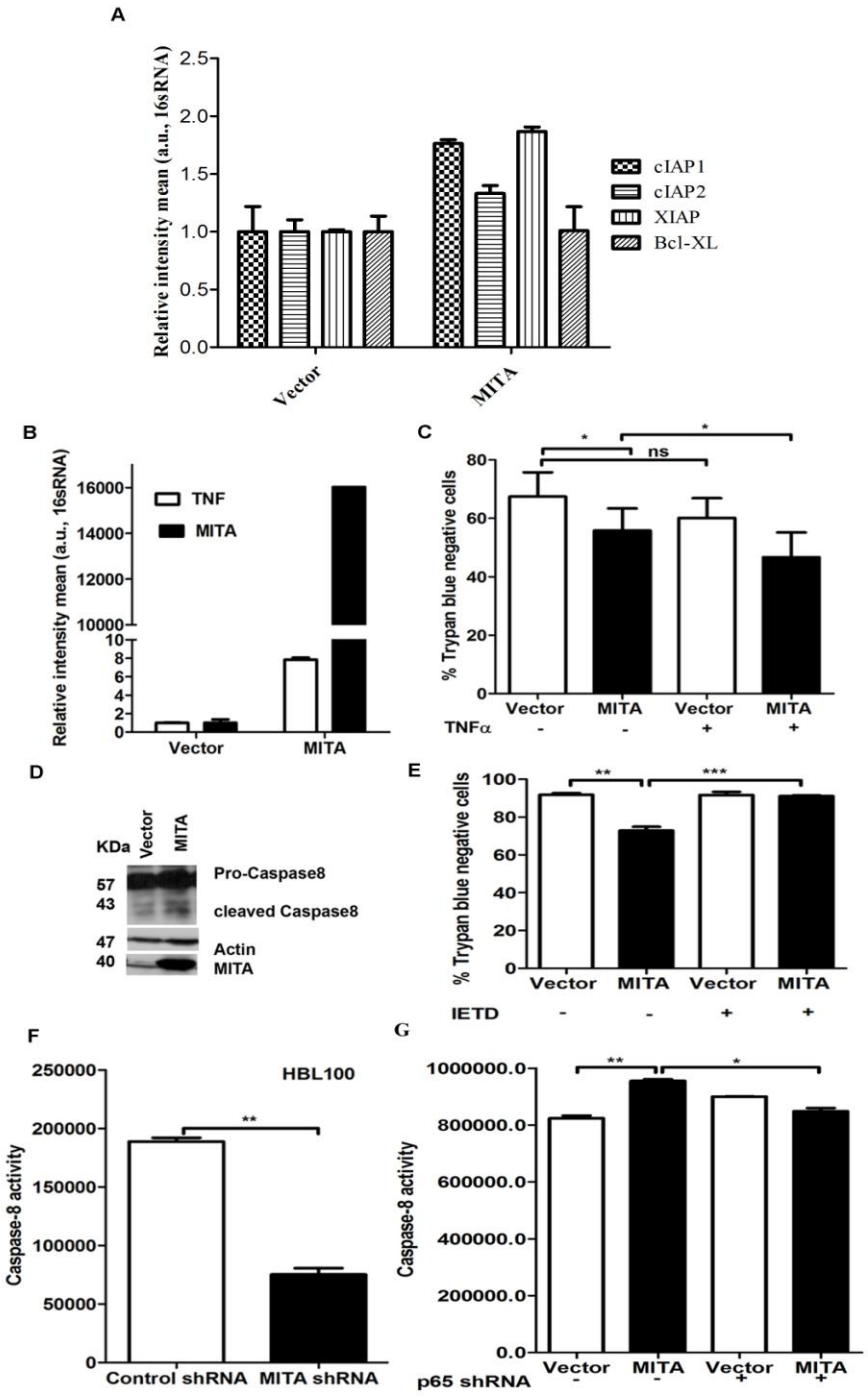

Figure-6.5 TNF-α mediated caspase-8 plays amajor role in MITA induced cell death: *MCF-7 cells were transfected with MITA. After 24 hrs of transfection, RNA was isolated and reverse transcribed to cDNA. NF-κB responsive genes expression (A) (BCL-XL, BCL-2, Bax, cIAP1, cIAP2 and XIAP) and (B) TNF-α was quantified using quantitative real time PCR. (C) MCF-7 cells were transfected with MITA and treated with TNFα 10 ng/ml for 24 hrs and cell death was monitored using trypan blue exclusion assay. (D) MCF-7 cells were transfected with MITA and caspase-8 activation was monitored. Cells were collected and lysed after 24 hrs of transfection and subjected to western blot analysis. Caspase-8 cleavage was analyzed using caspase-8 specific antibodies. (E) MCF-7 cells were transfected with MITA and treated with z-IETD-fmk (1μM) and cell death was monitored using trypan blue exclusion assay. (F) MITA shRNA was transfected in HBL100 cells and caspase-8 activation was monitored using caspase-8 luminescence based assay. (G) MCF-7 cells were cotransfetced with p65 shRNA and MITA and cell death was monitored using trypan blue exclusion assay.*

6.7. Discussion

Current study provides an essential link between inflammation and cancer. As described in chapter 2 inflammation and breast cancer are intricately linked to each other but the mechanism is not well understood. NF-κB and IFNs are the central regulators linking inflammation to cancer. The evidences suggest that MITA leads to cell death in breast cancer cell that is regulated by NF-κB. The cell death induction is evident from the results of decrease in MTT reduction, increase in trypan blue negative cells, cleavage of PARP and caspase 8 upon MITA expression. Inhibition of NF-κB can lead to increased sensitivity to apoptotic signals but this does not indicate NF-κB activation leads to cell survival. In fact, NF-κB is a dynamic transcription factor that regulates several genes including anti-apoptotic (Bcl-2, Bcl-X_l, XIAP, cIAP1, cIAP2) as well as pro-apoptotic genes (Fas, FasL, DR4, DR5) [126]. Simultaneous stimulation of death stimuli as well as NF-κB activation leads to apoptosis [130]. The current study emphasize the role of NF-κB in MITA regulated cell death and pro-apoptotic role of NF-κB. Dysregulation of NF-κB has been observed in various tumorous conditions [131, 132]. The association of NF-κB and breast cancer is further emphasized by the observation of amplification and over-

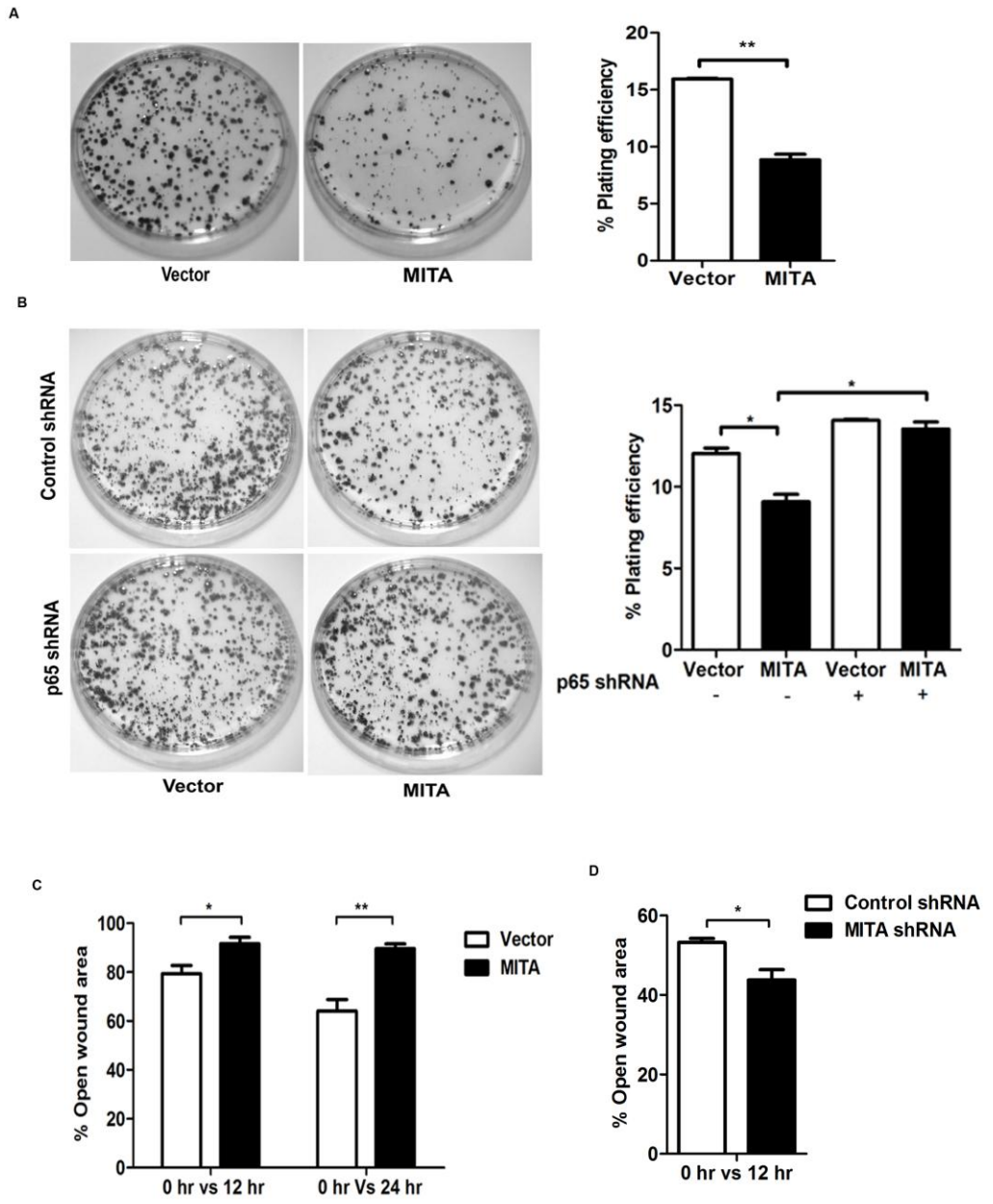

Figure-6.6: MITA regulates tumorigenic potential of breast cancer cells:

(A) MCF-7 cells were transfected with MITA and clonogenic ability of the cells was monitored using colony forming assay. (B) MCF-7 cells were cotransfected with p65shRNA and MITA and clonogenic ability of the cells was monitored using colony forming assay. (C) MITA was expressed in MCF-7 cells and migration ability of MCF-7 cells was monitored using scratch assay. (D) MITA shRNA was transfected in HBL100 cells and migration ability of MCF-7 cells was monitored using scratch assay.

expression of IKKε, a central kinase in NF-κB pathway, in breast cancer cell lines and patient-derived tumors[68]. Over expression of the gene is observed in 30% of the breast cancers cell lines and carcinomas[68]. It is also observed that IKKε translocates to nucleus in response to DNA damage as discussed in chapter-2. MITA is also observed to be responsive to dsDNA[19]. IKKε acts downstream to MITA in RIG-I pathway [17]. IKKε and MITA might also play a role together in response to dsDNA. These observations again give a connecting link to MITA, inflammation and breast cancer.

The expression studies showed up regulation of TNFα upon MITA expression in breast cancer cells. TNFα treatment further sensitized MITA induced cell death in breast cancer cells. TNFα leads to the activation of caspase-8. The inhibition of caspase experiment as well as caspase 8 activation by MITA expression further strengthen the role of TNFα in cell death induction through MITA. TNFα is already known to play a very important role in cancer as well as inflammation[133]. It is indeed given as a tumor therapeutic in combination with melphalan for the treatment of advanced soft tissue sarcoma of the extremities[134]. One of the recent studies has shown that TNFα expressing MDA-MB231 cells failed to form tumor in vivo[135]. It also suggests that TNFα interrupts symbiotic metabolic coupling between epithelial cancer cells and their host stromal microenvironment[135]. This prevents metabolic symbiosis between tumor and its microenvironment which effectively starves the cancer cells to death. MITA down regulation in breast cancer tissue is strategy of tumor to resist the antitumor effect of TNFα. Intrestingly TNFα is also known to play important role in regulation of mitochondrial function therefore in normal conditions the MITA regulated TNF may regulate mitochondrial bioenergetics to control the process of tumorigenesis.

Evidences show that expression of MITA leads to decrease in clonogenic ability as well as in migration ability of MCF-7. The migration ability of MCF-7 as well as HBL100 cells is also affected in the presence or absence of MITA respectively. The decrease in the migration ability of MCF-7 and increase in the caspase 8 activity upon MITA expression might have an interesting link to study. The studies have shown that caspase 8 binds to the lamella of the migrating cell and promotes the cell migration. Caspase 8 catalytic activity is not required in the process though. Tyrosine kinases phosphorylates tyrosine 380 of caspase 8 which inhibits further maturation of the caspase[136]. The binding of caspase 8

to lamella of migrating cells promotes the cell migration[137]. In fact mutation in Tyr380 affects the migration potential[137]. The decreased migration of MCF-7 cells upon MITA expression might be due to the increase in caspse8 activity which ultimately makes pro-caspase8 unavailable to aid in migration. Unraveling the mechanism of role of MITA in connecting these two observations would be really interesting to study.

In conclusion, our study unfolds the role of MITA as a tumor suppressor gene in breast cancer. MITA is significantly down regulated in breast cancer patients also in ER positive breast cancer cell line. MITA regulates the cell death by induction of NF-κB. NF-κB further up regulates TNF which sensitizes MITA induced cell death. Thus, MITA protein might prove to be an essential link to inflammation, mitochondria and cancer but it needs further research in this area. This hypothesis clearly warrants further study to understand link between inflammation and breast cancer.