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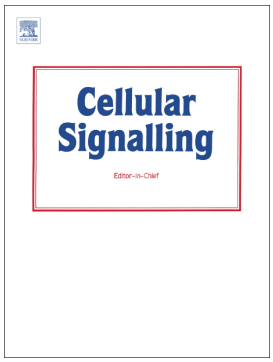

MITA modulated autophagy flux promotes cell death in breast cancer cells

Khyati Bhatelia, Kritarth Singh, Paresh Prajapati, Lakshmi Sripada, Milton Roy, Rajesh Singh

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Title: MITA modulated autophagy flux promotes cell death in breast cancer cells

Khyati Bhatelia[1], Kritarth Singh[1], Paresh Prajapati[1], Lakshmi Sripada [1], Milton Roy[1], Rajesh Singh[1#]

Author's Affiliation:

[1]Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Sayajigunj, Vadodara -390002, Gujarat, India.

[*]**Running Title:** MITA regulates autophagy in breast cancer cells

[#]**To whom correspondence should be addressed:** Dr. Rajesh Singh, Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara - 390002, Gujarat, India. Tel- +91-265-2795594 Email: singhraj1975@gmail.com.

Abstract:

The crosstalk between inflammation and autophagy is an emerging phenomenon observed during tumorigenesis. Activation of NF-κB and IRF3 plays a key role in the regulation of cytokines that are involved in tumor growth and progression. The genes of innate immunity are known to regulate the master transcription factors like NF-κB and IRF3. Innate immunity pathways at the same time regulate the genes of the autophagy pathway which are essential for tumor cell metabolism. In the current study, we studied the role of MITA (Mediator of IRF3 Activation), a regulator of innate immunity, in the regulation of autophagy and its implication in cell death of breast cancer cells. Here, we report that MITA inhibits the fusion of autophagosome with lysosome as evident from different autophagy flux assays. The expression of MITA induces the translocation of p62 and NDP52 to mitochondria which further recruits LC3 for autophagosome formation. The expression of MITA decreased mitochondrial number and enhances mitochondrial ROS by increasing complex-I activity. The enhancement of autophagy flux with rapamycin or TFEB expression normalized MITA induced cell death. The evidences clearly show that MITA regulates autophagy flux and modulates mitochondrial turnover through mitophagy.

Highlights:

- MITA negatively regulates autophagy flux in breast cancer cells.
- MITA regulates mitochondrial homeostasis by regulating mitophagy and PGC1α.
- Increased level of MITA enhances ROS in breast cancer cell.
- Enhancement of autophagy rescues breast cancer cells from MITA induced cell death.

Keywords: MITA, Autophagy flux, mitophagy, inflammation, breast cancer

1. **Introduction**

The tumor microenvironment is complex milieu having the cells of different origin, including immune cells [1, 2]. The tumor cells show increased levels of definite pattern of cytokines which probably helps the tumor cells to reprogramme gene expression pattern and metabolism for their survival [3, 4]. Interestingly, the origin of the increased level of cytokines is attributed to the immune cells that are recruited to the tumor microenvironment. The role of the tumor cells themselves in regulation of inflammation is not yet clear and needs to be established in order to modulate different metabolic and signaling pathways to inhibit tumor cells proliferation and metastasis.

NF-κB and IFNs are key regulators of distinct set of anti- and pro-inflammatory cytokines. The regulators of these pathways are critical in different innate immune pathways. It is observed that during the cellular transformation, the genes regulating antitumorigenic cytokines are lost from the tumor cells. MITA, for example, is downregulated or functionally inactivated in different types of cancer including breast cancer, acute myeloid leukemia and prostate cancer [5-7]. Interestingly, the adaptor proteins like MAVS and MITA, antiviral signaling proteins, are localized on the mitochondria and ER-mitochondria contact site respectively [8, 9]. During viral infection, RNA and DNA viruses are recognized by distinct proteins like RIG1 and cyclic GMP-AMP synthase (cGAS) respectively which further interact with MITA [10, 11]. The interaction recruits downstream signaling proteins and activate NF-κB and IFN that induces anti viral response. Moreover, the evidences also suggest that these proteins regulate mitochondrial functions during infection [12]. The specific localization of these proteins on mitochondria suggests their role beyond innate immunity in metabolism under normal physiological conditions.

It is observed that critical innate immunity pathways (NF-κB and IFN) are also involved in the regulation the expression of the genes involved in autophagy [13-15]. NF-κB regulates the expression of p62 that is essential for the regulation of selective elimination of defective mitochondria called as mitophagy [13]. The increased level of autophagy is important for the tumor cell metabolism and adaptation for increased rate of cell division [16, 17] . The selective elimination of the defective organelles via autophagy is important for the cellular homeostasis [18-20]. The degradation of mitochondria may down regulate the levels of several mitochondrial and mitochondrial associated membrane resident proteins like MAVS and MITA hence downregulating and maintaining inflammation in physiological limits.

Autophagy (macroautophagy) is a sequential process of degradation of cytoplasmic material as well as organelles through lysosomes. The first step involves the formation of autophagophore membrane which encloses the portion of cytoplasm to form autophagosome[21]. The outer membrane of autophagosomes fuses with lysosomes and forms autophagolysosomes [22, 23]. The lysosomal enzymes degrades the enclosed cytoplasmic material and inner membrane of autophagosome [24]. Defect in autophagy leads to several diseased conditions including neurodegeneration, inflammatory diseases and cancer [25]. TFEB (Transcription factor of TFEB) is a transcription regulator of CLEAR network of lysosomal biogenesis [26]. TFEB also regulates autophagic flux by regulating genes involved in autophagosome synthesis, cargo recognition, autophagosome to lysosome fusion and degradation [27].

In our previous report, we have demonstrated that MITA acts as tumor suppressor and is lost during breast cancer cell transformation [6]; however its mechanism is still not well understood. In the current study, we demonstrate that MITA regulates the process of autophagy at the autophagosomal-lysosomal fusion step. The turnover of mitochondria through mitophagy is

inhibited in the presence of MITA. The defective mitophagy leads to accumulation of damaged mitochondria and increased level of ROS and cell death. Enhancement of autophagy rescues the breast cancer cells from MITA induced cell death.

2. Materials and methods:

2.1 Cells and Cell Culture

MCF-7 breast cancer cell line was obtained from National Center for Cell Sciences, Pune, India. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, USA). T47D cells were cultured in RPMI 1640 (Life Technologies, USA). The media used were supplemented with 10% FBS (Life Technologies, USA) and 1% penicillin, streptomycin, and neomycin (PSN) antibiotic mixture (Life Technologies, USA). Cells were incubated at 37°C, 5% CO_2 in specified media.

2.2 Plasmids and Reagents

MITA cloned in pCMV6-ENTRY plasmid was a gift from Dr. Hong-Bing Su (Wuhan University, China). Primary antibodies used were MITA (Proteintech, USA), PARP and NDP52 (Cell Signaling Technology, Inc, USA), β-Actin (Abcam, USA), TFEB and GFP(Abclonal Technology, USA), LC-3 (Sigma-Aldrich, USA) and p62(GeneTax, USA). HRP-conjugated anti-rabbit and anti-mouse antibodies (Thermo Scientific, USA) were used. The reagents used were Rapamycin, NH_4Cl and Bafillomycin (Sigma-Aldrich, USA). TFEB-GFP was provided by Prof. Andrea Ballabio (Scientific Director, TIGEM) [28]. LC3-GFP was provided by Dr. T. Yoshimori (National Institute of Genetics, Shizuoka, Japan)[29], LC3-mCherry-GFP, p62-mCherry- GFP and p62-GFP by Dr. Terje Johansen (Dept. of Biochemistry, Institute of Medical Biology, University of Tromsø)[30]. MITA-mcherry (mouse) and MITA-mcitrine (human) constructs were gifted by Dr. Veit Hornung (University Hospital of Bonn, Sigmund-Freud-Strasse 2553127 Bonn, Germany)[31].

2.3 Transfection

MCF-7 cells were transfected using Biotool DNA transfection reagent (Biomake, USA) as per the manufacturer's protocol.

2.4 Quantitative analysis of gene expression

Total RNA was isolated using Tri Reagent (Life Technologies, USA). cDNA was synthesized using Primescript First Strand cDNA synthesis kit (Takara, Japan) according to the manufacturer's instructions. Real time PCR was performed using SYBR Premix Ex TaqTM (Takara, Japan) as per the manufacturer's instructions.

2.5 Analysis of Reactive Oxygen Species (ROS) and mitochondrial potential:

Cells were plated in 12-well plate at the density of $2X10^5$cells/per well. Cells were transfected with MITA as well as vector control. Intracellular ROS level was measured by CM-H_2DCFDA (10 μM) and mitochondrial potential was measured using tetramethylrhodamine, methyl ester (TMRM). The cells were washed thrice using DPBS. ROS levels were quantified by spectrofluorometric analysis using F-7000 Fluorescence Spectrophotometer (Hitachi, Japan).

2.6 Sub cellular fractionation:

Mitochondrial [32] and nuclear fractionation [6] analysis were performed as described in our previous reports.

2.7 Western Blot Analysis:

Cells were plated at a density of 5×10^5 cells/well in the six well plate and transfected with indicated expression plasmid. After 48 hr of transfection, the cells were harvested, washed with

ice cold PBS and lysed in buffer A (150 mM NaCl, 30 mM Tris–Cl, 10% Triton X-100, 10% Glycerol, 1X Protease Inhibitor (Roche, Germany). The equal protein was loaded and resolved on 11% SDS-PAGE. Protein was electroblotted on PVDF membrane at 110 V for 1 h at 4 °C. The membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS) or 5% BSA (BSA (Sigma-Aldrich, USA), 0.1 % Tween-20 in TBS-0.02M Tris-Cl, 0.15M NaCl) for 1 h at room temperature. The membrane was incubated overnight with specific primary antibody. After incubation, the membrane was washed three times with TBS-T (TBS containing 0.1% Tween 20) for 10 min and incubated with a secondary antibody at room temperature for 1 h. The membrane was washed three times with TBS-T and signal was visualized by using EZ-ECL chemiluminescence detection kit for HRP (Biological Industries, Israel) by exposing it to X-ray film.

2.8 Complex-I Activity Assay:

Mitochondrial complex-I activity was analyzed spectrophotometrically as described previously [32].

Complex-I activity was also performed using In-gel activity assay. The cells were plated in 60 mm dish at density of 5 X 10^6 and cultured for 24 hours. The cells were transfected with specific constructs and complex-I activity was determined by BN-PAGE (Blue-Native Page). BN-PAGE was performed as described previously [33]. The gel was stained with complex-I staining solution (50 mM potassium phosphate buffer (pH 7.0) containing 0.2 mg/ml NBT and 0.1 mg/ml NADH).

2.9 ATP assay:

Total ATP measurement was performed using ATP Bioluminescence kit CLS II (ThermoFisher Scientific, USA) as per the manufacturer's protocol.

2.10 Caspase 3/7 Activity:

Caspase-3/7 activation assay was performed using Caspase-Glo® 3/7 Assay Systems (Promega, USA) as per the manufacturer's protocol.

2.11 Microscopic Analysis:

Cells were plated on coverslips and transfected with specified constructs. After 36 h of transfection, cells were fixed with 4% para-formaldehyde and stained with DAPI. The cells were analyzed by Leica DMI 8000 fluroscent microscope for LC3-GFP puncta analysis at 40X. All the other experiments were analyzed using Leica SP8 confocal microscope (Leica Microsystems, Germany) by sequential imaging using 63X objective with 3X zoom (Confocal microscopy).

2.12 Statistical analysis:

Data are shown as mean ± SEM for number of times the experiment was repeated. Comparisons of groups were performed using student t-test for repeated measurements to determine the levels of significance for each group. The experiments were performed minimum two times independently and $p<0.05$ was considered as statistically significant. GraphPad Prism software was used to perform all the statistical analysis.

3. **Results:**

3.1 MITA inhibits the autophagy flux in breast cancer cells:

The high level of autophagy in tumor microenvorinment is essential to meet the increased nutrient demand of proliferating cells[16, 34-36]. MITA enahnces NF-κB, which regulates the expression of key proteins regulating autophagy[13] hence we analyzed if MITA is essential for the regulation of autophagy in breast cancer cells. As observed in our previous study, the expression of MITA was downregulated in most of the breast cancer lines as compared to MCF-10A[6]. The expression of MITA was not detected in MCF-7 cells and therefore selected for futher study. MITA was over expressed in MCF-7 cells and its role in induction of autophagy was monitored. The cells were analysed for autophagy induction by LC3 western blotting. The increased levels of 16 kDa band corresponding to LC3-II was observed in MITA expressed cells as compared to control cells (Fig 1a). Autophagy induction was also monitored by monitoring the LC3-puncta using fluroscent microscopy. MCF-7 cells were co-transfected with LC3-GFP and MITA and LC-3 puncta formation were analyzed using fluroscent microscope. The number of LC-3 puncta significantly increased in MITA transfected cells (Fig 1b and c). As described, p62 is an adaptor protein of autophagy; hence p62 puncta formation was also analyzed in the presence of MITA. The number of p62 puncta increased significantly in the presence of MITA (Fig 1d and e) as compared to vector transfected cells. These results suggest that expression of MITA increased the number of autophagosomes in MCF-7 cells.

The formation of autophagosome is generally, but not always, an indicator of the level of cellular autophagic activity[24]. It is further essential to monitor if autophagic flux is normal and autophagosomes fuses with lysosome and degraded in normal cellular conditions. The cells were

treated with bafilomycin to monitor autophagy flux. Bafilomycin A1 is a specific inhibitor of V-ATPase; hence it inhibits autophagosome and lysosome fusion and induces lysosomal dysfunction by increasing the pH of lysosome[37]. The inhibition of autophagy using bafillomycin stabilized both the forms of LC-3 (LC-3-I/II) but the level of 16 kDa band corresponding to LC3-II was same in control as well as MITA transfected MCF-7 as well as T47D cells (Fig-2a and b). Moreover, p62 (another marker of autophagy) levels also increased in MITA transfected T47D cells. The levels of p62 further increased with bafilomycin treatment (Fig-2b). This suggests that autophagosomes are not delivered to lysosomes for degradation or lysosomal activity is not optimal. To further confirm the flux, LC3-GFP cleavage assay was performed. LC3-GFP fusion protein is cleaved in the lysosomes releasing free GFP which can be detected by immunoblotting. The assay monitors the functionality of lysosomes. MITA was cotransfected with LC3-GFP fusion protein and GFP cleavage was monitored by western blotting. GFP cleavage was inhibited in the presence of MITA (Fig 2c). The level of free GFP increased in the presence of rapamycin (inducer of autophagy) whereas it decreased in the presence of NH_4Cl (inhibitor of lysosomal pH). The evidences thus suggest the defect in autophagy flux in MITA expressing cells. The flux was also analyzed using LC3/p62-GFP-mcherry tandem construct. The tandem construct has GFP and mcherry tagged to LC3/p62 which flurosce yellow/oragne in autophagosome. The autophagosomes once fuses with lysosomes, the GFP fluroscence is quenched in acidic pH conditions of lysosomes and mature autolysosome are detected as red puncta[24]. The tandem construct of LC3-mcherry-GFP was cotransfected with MITA and number yellow puncta were counted. Number of yellow LC3 puncta increased signficantly as compared to red pucta in MITA transfected cells (Fig 3 a and c). Similarly, cells co-expressing MITA and p62-mcherry-GFP tandem construct showed increased yellow colored

puncta as compared to red puncta (Fig 3 b and d). In positive control, rapamycin treated cell showed significant increase in red color puncta (Fig 3 a-d). These evidences suggest that MITA negatively regualtes the fusion of autophagosome to lysosome.

3.2 MITA inihibts the turnover of mitochondria through mitophagy:

The inhibtion of autophagy flux by MITA may lead to accumulation of defective organelle or reduced nutritent turnover which may negatively affect the tumor growth. The accumulation of defective mitochondria leads to oxidative stress mediated cell death; hence we further analyzed mitophagy in the presence of MITA. Mitophagy was analyzed using LC3-II recruitment on mitochondria. We observed increased level of 16kDa band corresponding to LC3-II in mitochondrial fraction of MITA expressing cells as compared to vector transfected cells. There was no significant difference in the level of LC3-II band obsseved in MITA or vector transfected cells in the presence of NH_4Cl (Fig 4a). During mitophagy, NDP52 and p62 are recruited on mitochondria to initiate the formation of autophagosome biogenesis hence are considered as marker of mitophagy [38, 39]. The level of NDP52 and p62 also increased in mitochondrial fraction in MITA transfected cells as compared to vector transfected cells. During normal conditions, autophagosomes containing mitochondria (mitophagosomes) are targeted to lysosome and degraded along with adaptor proteins such as p62 and NDP52[40]. Intrestingly, the 52 or 62 kDa band corresponding to NDP52 and p62 increased in mitochondrial fraction in MITA expressing cells as compared to vector control cells (Fig 4a). LC3-II and p62 recruitment on mitochondria was further confirmed with confocal microscopy analysis. LC3-GFP/p62-GFP were cotransfected with MITA and mitochondria were stained with TMRM stain. The recruitment of green p62 puncta on red mitochondria was evident from confocal microscopic analysis (Fig 4b). The increased number of green-p62 puncta and its colocalization with

mitochondria was observed in the presence of MITA (Fig 4c). Similarly number of green-LC3 puncta increased and colocalized on red mitochondria in the presence of MITA (Fig 4d and e). These evidences suggest that adaptor proteins p62/NDP52 further recruits LC3 on mitochondria in the presence of MITA. The mitophagosomes however are not targeted to lysosome for degradation in the presence of MITA because of the defective flux.

The defective flux might affect the mitochondrial biogenesis or turnover. PGC1α is a transciptional activator and stimulator of mitochondrial biogenesis[41]. The expression of PGC1α was hence analyzed in MITA trasfected cells. The levels of PGC1α significantly decreased in MITA transfected cells (Fig 5a) as compared to vector. NRF1 and NRF2 are also transcriptional regulator of mitochondrial biogenesis [41, 42] and act downstream of PGC1α; hence their levels were also analyzed. The increased level of NRF-1 was observed in MITA transfected MCF-7 cells (Fig 5b) whereas no difference was observed in case of NRF-2 (Fig 5c). Downregulation of PGC1α suggested that mitochondrial biogenesis is inhibited. Hence to further confirm the same, we analyzed the level of mitochondrial DNA. Significant decrease in mitochondrial DNA content was observed in the presence of MITA (Fig 5d). These evidences suggest that expression of MITA downregulates mitophagy and inhibits the mitochondrial biogenesis creating bioenergetic crisis in breast cancer cells.

3.3 MITA regulates mitochondrial function in MCF-7 cells:

The decrease in autophagy flux in presence of MITA leads to accumulation of damaged mitochondria and generates ROS in the breast cancer cells [43]. This activates RIGI signaling pathway and further leads to increased level of type-I- IFN hence we further analyzed mitocondrial functions in presence of MITA [43]. ROS generation was monitored using the oxidant-sensitive dye, CM-H_2DCFDA. Interestingly, MITA expression significantly increased

ROS levels as compared to control cells (Fig 6a). The mitochondrial complex-I of the electron transport chain is a major site of electron entry and ROS generation [44]; hence we hypothesize that MITA may regulate complex-I activity. Mitochondrial complex-I activity was analyzed using oxidation of NADH by spectrophotometer and BN-PAGE (Fig 6b and c). The complex-I activity increased in MITA expressing cells as compared to control. Mitochondrial membrane potential was measured using TMRM stain and it decreased in the presence of MITA (Fig 6d). The ATP levels also decreased in the presence of MITA as compared to control (Fig 6e). These evidences suggest that the defect in mitophagy flux leads to accumulation of dysfunctional mitochondria.

3.4 Enhancement of autophagy flux rescues from MITA induced cell death:

We further hypothesized that enhancement of the autophagic flux by rapamycin may rescue MCF-7 cells from MITA induced cell death. MITA transfected cells were hence treated with rapamycin and caspase activity was analyzed in MCF-7 cells. The expression of MITA increased caspase activity, which decreased in the presence of rapamycin (Fig 7a). TFEB is considered as the master regulator of the genes involved in lysosomal biogenesis and autophagy pathway [26]. Rapamycin induces nuclear translocation of TFEB to enhance autophagy and lysosomal functions [26]; hence, we analyzed the nuclear translocation of TFEB using confocal microscopy. We observed increased nuclear translocation of TFEB in the presence of MITA (Fig 7b and c). We further assessed caspase activity of MITA and TFEB co-transfected MCF-7 cells. The transfection of MITA enhanced the caspase activity; whereas, it was normalized in TFEB and MITA cotransfected cells (Fig 7d). The evidences in the current study showed that MITA negatively regulates autophagy flux and hence mitophagy. Enhancement of the autophagy flux using rapamycin and TFEB rescued breast cancer cells from MITA induced cell death.

4. **Discussion:**

Inflammation, autophagy and cellular transformation are emerging trio that is critical for development of tumor [45]. It has been observed that during the course of cellular transformation, either there is complete lose or mutation of the tumor suppressors and amplification of oncogenes for successful transformation. Similarly, the genes regulating innate immune response during viral and bacterial infections are also expressed in normal breast epithelium. We observed previously that MITA regulating type-I IFN and NF-κB act as tumor suppressor and its expression is reduced significantly in the breast cancer tissue as well as breast cancer cells [6]. In this study we convincingly demonstrate that MITA negatively regulates autophagy flux and its implication in cell death in breast cancer cells.

Recent reports strongly suggest that activation of IFN and NF-κB regulate some critical genes involved at different steps of autophagy [13, 15]. The process of autophagy is initiated through autophagosome biogenesis and the origin of autophagosome membrane still remains controversial; however, ER is considered as the potential source [46]. MITA is localized on ER; hence we further hypothesized that it may regulate autophagosome biogenesis. The increased level of conjugated LC3-II band and LC3/p62 puncta in the cells observed in the current study suggest the increased number of autophagosomes. The flux experiments however suggest that the fusion of autophagosome to lysosome is blocked. Consistently increased yellow colored puncta was observed in tandem-GFP-RFP construct and decreased p62 degradation in MITA co-transfected cells. These results suggest that MITA may regulate the autophagy flux in normal conditions; whereas, the reduced expression of MITA in cancer tissue may provide survival advantage by enhancing the autophagy flux.

The defect in autophagy process leads to accumulation of defective mitochondria which may negatively regulate the tumor cell metabolism [16, 17], [47] and tumor progression. In MITA transfected cells, the adaptor proteins having LC3 interaction region, like p62/NDP52 are recruited on mitochondria. This further recruits LC3 and initiates elongation of phagophore around the mitochondria. The further experiments suggest that mitophagosome (autophagosome containing mitochondria) fusion to lysosome is defective; hence, leading to accumulation of defective mitochondria. The expression of MITA showed increased complex-I activity; however decreased levels of ATP suggesting the decrease in electron flux. ROS may be generated due to the reverse electron transfer in complex-I which may have role in amplifying NF-κB activity. It had been observed earlier that mitochondrial complex-I generated ROS activates NF-κB that helps in differentiation of muscle cells [48]. Similarly in recent report it had been observed that mitochondrial ROS is generated during intestinal stem cells (Lgr5+ crypt base columnar cells (CBCs) into mature crypt cell [49]. This further supports our hypothesis of MITA as potential regulator of mitochondrial functions and ROS regulator in normal conditions. Moreover, ROS are also an integral part of TNF mediated cell death [50]. MITA is also known to sensitize TNF mediated cell death as discussed in our previous report[6]. The generation of ROS might further amplify mitochondria mediated cell death pathway. The accumulation of mitochondria may also provide extra ER-mitochondria contact sites providing platform for the assembly of signaling complex of MITA which may further increase the level of type-I IFN [8]. Tumor inhibitory potential of Type-I IFNs is well known and have been used to treat haematological malignancies as well as solid tumors [51]. This further supports that MITA acts as potent tumor suppressor in breast cancer [6] as well as other tumors [5, 7].

Furthermore, MITA downregulates PGC-1α expression and inhibits further mitochondrial biogenesis. PGC1-α is known to regulate mitochondrial biogenesis as well as oxidative phosphorylation [52]. Metabolic reprogramming is an integral part of cancer cells to promote enhanced rate of proliferation[53]. Cancer cell generally shifts the metabolism towards aerobic glycolysis to support their growth; however, they simultaneously rely on mitochondrial TCA cycle and ETC for further anaplerotic reactions [54]. Reports also suggest strong correlation between high levels of PGC-1α and increased metastasis in breast cancer cells [52]. Our observation also suggests decrease in PGC1α levels in the presence of MITA. PGC1α though does not directly bind to DNA, it binds to transcription factors such as NRF-1 and NRF-2 and leads to increased mitochondrial biogenesis [42, 55]. The reduced levels of PGC1α decreases mitochondrial biogenesis in spite of increased levels of NRF1 as it acts upstream of NRF-1 and NRF-2. This also suggests that MITA regulated expression of PGC1α may be responsible for distinct pattern of gene expression involved in the tumor suppressor mechanisms. This hypothesis is supported by a recent report which suggests that reduced expression of PGC1α suppresses melanoma metastasis, acting through a pathway distinct from that of its bioenergetic functions[56]. Therefore, it will be interesting to further study the different role of MITA in tumor suppressive mechanisms. The restoration of MITA expression in MCF-7 cells, on one hand leads to accumulation of damaged mitochondria and on the other hand inhibits replenishment of healthy mitochondria to cell. This creates situation of bioenergetic crisis leading to cell death. The type of cell death initiated by MITA needs to be validated.

Further we hypothesized that if the block in autophagsome/lysosome fusion is rescued either by enhancing autophagy flux or enhancing lysosomal capacity, MITA induced cell death can be rescued. The transcription factor EB (TFEB) plays a critical role in lysosomal biogenesis and

modulating autophagy dynamics. The subcellular localization and activity of TFEB are regulated by mechanistic target of rapamycin (mTOR)-mediated phosphorylation, which occurs at the lysosomal surface. During starvation or mTOR inhibition by rapamycin , TFEB translocates to the nucleus and activates genes regulating synthesis of autophagosomes and lysosomes [26, 27, 57]. The treatment of MITA transfected cells with rapamycin showed decreased caspase activity. Similarly, over expression of TFEB rescued MITA induced cell death. This suggests that expression of TFEB rescued lysosomal biogenesis as well as genes of the autophagy pathway which establishes the autopahgy flux in presence of MITA.

5. Conclusion

The current evidences suggest that MITA regulates autophgosomal/lysosomal fusion step to downregulate mitophagy. The study here suggest that MITA mediated regulation of autophagy flux may lead to accumulation of defective mitochondria. This may be selective mechanisms for induction of mitochondrial ROS that may either lead to cell death or amplify differentiation process as described earlier [49, 58, 59]. Moreover, damaged mitochondria might release mitochondrial DNA inside the cytoplasm, which may act as DAMPs and activate cGAS/MITA mediated type-I IFN production [60-62]. The activation of this pathway can be of potential in immunotherapy of breast cancer. The study here further establishes MITA as tumor suppressor by regulating mitophagy and autophagy flux in breast cancer cells.

Conflict of interest

There is no conflict of interest to declare.

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Figure Legends:

Figure-1: Expression of MITA leads to the accumulation of autophagosomes:

(A) MCF-7 cells were transfected with MITA and LC3 levels were analyzed by western blotting. (B) MCF-7 cells were cotransfected with LC3-GFP and mMITA-mcherry. The autophagosome formation was observed as green puctate structure using fluorescent microscopy. (C) Quantification of number of LC3 puncta formed inside the cells (individual cell). Numbers of autophagosomes (green puncta) per cell were counted in minimum 30 cells and graph was plotted for number of LC3 green puncta per cell of representative Fig-1B. (D) MCF-7 cells were cotransfected with p62-GFP and MITA. The autophagosome formation was detected as green puctate structure using confocal microscopy. (E) Quantification of number of p62 pucta formed inside the cells. Numbers of autophagosomes (green puncta) per cell were counted in minimum 40 cells and graph was plotted for number of LC3 green puncta per cell of representative Fig-1D.

Figure-2: The expression of MITA inhibits the autophagic flux:

(A) MCF-7 cells were transfected with MITA and treated with Bafilomycin to monitor the autophagic flux. LC-3II levels were analyzed by western blotting. (B) T47D cells were transfected with MITA and treated with Bafilomycin to monitor the autophagic flux. LC-3II and p62 levels were analyzed by western blotting. (C) MCF-7 cells were co-transfected with LC3-GFP and MITA and GFP cleavage analysis was performed by western blotting.

Figure-3: Rapamycin rescue MITA inhibited autophagosome fusion with lysosome in MCF-7 cells

(A) MCF-7 cells were cotransfected with LC3-mcherry-GFP tandem construct and hMITA-mcitrine in the presence or absence of rapamycin or NH_4Cl. The cells were analyzed by confocal

microscope for red and yellow puncta formation. (B) MCF-7 cells were cotransfected with p62-mchery-GFP tandem construct and hMITA-mcitrine in the presence or absence of rapamycin or NH_4Cl and cells were analyzed by confocal microscope yellow puncta. (C) Quantification of the autophagy flux. Numbers of autophagosomes (yellow puncta) per cell were counted in minimum 20 cells and graph was plotted for number of LC3-yellow puncta per MITA transfected cell of representative Fig-3A. (D) Quantification of the autophagy flux. Numbers of autophagosomes (yellow puncta) per cell were counted in minimum 20 cells and graph was plotted for number of p62-yellow puncta per MITA transfected cell of representative Fig-3B.

Figure-4: MITA inhibits the turnover of mitochondria through mitophagy:

(A) The cells were transfected with MITA and treated with NH_4Cl. The cells were lysed, subcellular fractionations were performed and mitochondrial fraction was subjected to western blot analysis. LC3-II, NDP52 and p62 levels were monitored in the presence of MITA. (B) MCF-7 cells were cotransfected with hMITA-mcitrine and p62-GFP. Mitochondria were further stained with TMRM. The colocalization of p62 with mitochondria were analyzed using confocal microscope. (C) The number of green punctarecruited on mitochondria were counted in minimum of 40 cotransfected cells and graph was plotted. (D) MCF-7 cells were cotransfected with hMITA-mcitrine and LC3-GFP. Mitochondria were further stained with TMRM. The colocalization of p62 with mitochondria were analyzed using confocal microscope. (E) The number of green puncta colocalized with mitochondria were counted in minimum of 15 cotransfected cells and graph was plotted.

Figure-5: MITA inhibits mitochondrial biogenesis by regulating PGC1α:

(A) MCF-7 cells were transfected with MITA and after 36 hrs of transfection RNA was isolated from cells, cDNA was prepared and (A) PGC-1α levels, (B) NRF-1 levels (C) NRF-2 levels were quantified using quantitative real time PCR analysis. (D) MCF-7 cells were transfected with MITA and total cellular DNA was isolated. mtDNA content was assessed by quantification of a unique mitochondrial fragment relative to a single copy region of the nuclear gene RNase P.

Figure-6: MITA regulates mitochondrial functions:

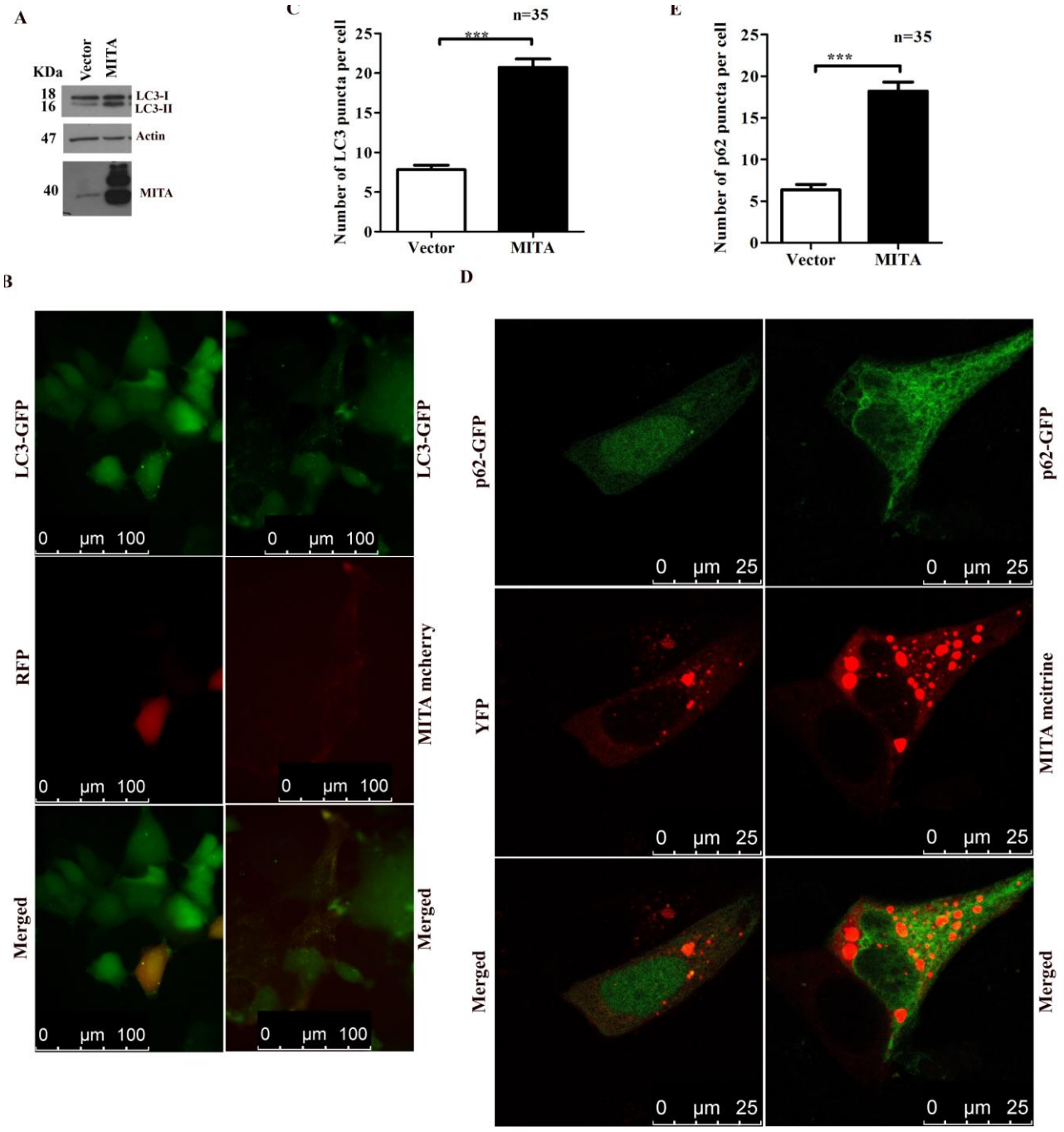
(A) MCF-7 cells were transfected with MITA. After 48 hours of transfection cells were stained with CM-H_2DCFDA and ROS levels were measured using flurometric analysis. (B) MCF-7 cells were transfected with MITA and complex-1 activity was analyzed spectrophotometrically and (C) in gel activity assay. (D and E) MCF-7 cells were transfected with MITA. After 48 hours of transfection cells, mitochondrial potential were measured by staining with TMRM (D) and cellular ATP levels were measured using luminometer (E).

Figure-7: Enhancing autophagy flux rescues MITA induced cell death

(A) The cells were transfected with MITA. After 24 hours of transfection cells were treated with rapamycin for 24 hours and caspase activity was measured using luminometer. (B) MCF-7 cells were cotransfected with hMITA-mcitrine and TFEB-GFP and subcellular localization of TFEB was analyzed using confocal microscopy in MITA transfected cells. (C) The number of cells showing nuclear translocation of TFEB was counted and the graph was plotted. (D) The cells were cotransfected with MITA and TFEB-GFP. After 48 hours of transfection caspase activity was measured using luminometer.

Figures:

Fig-1



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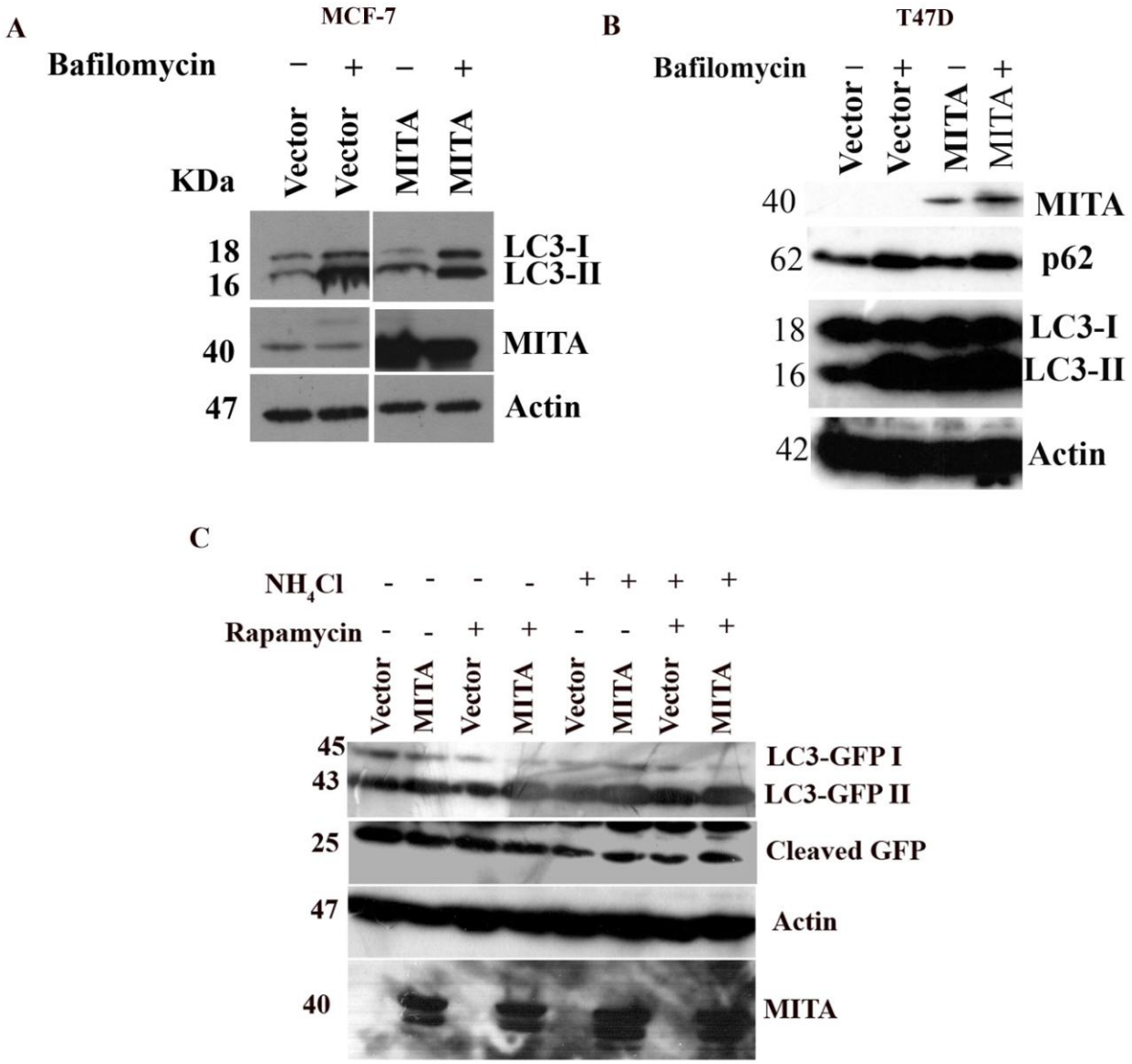

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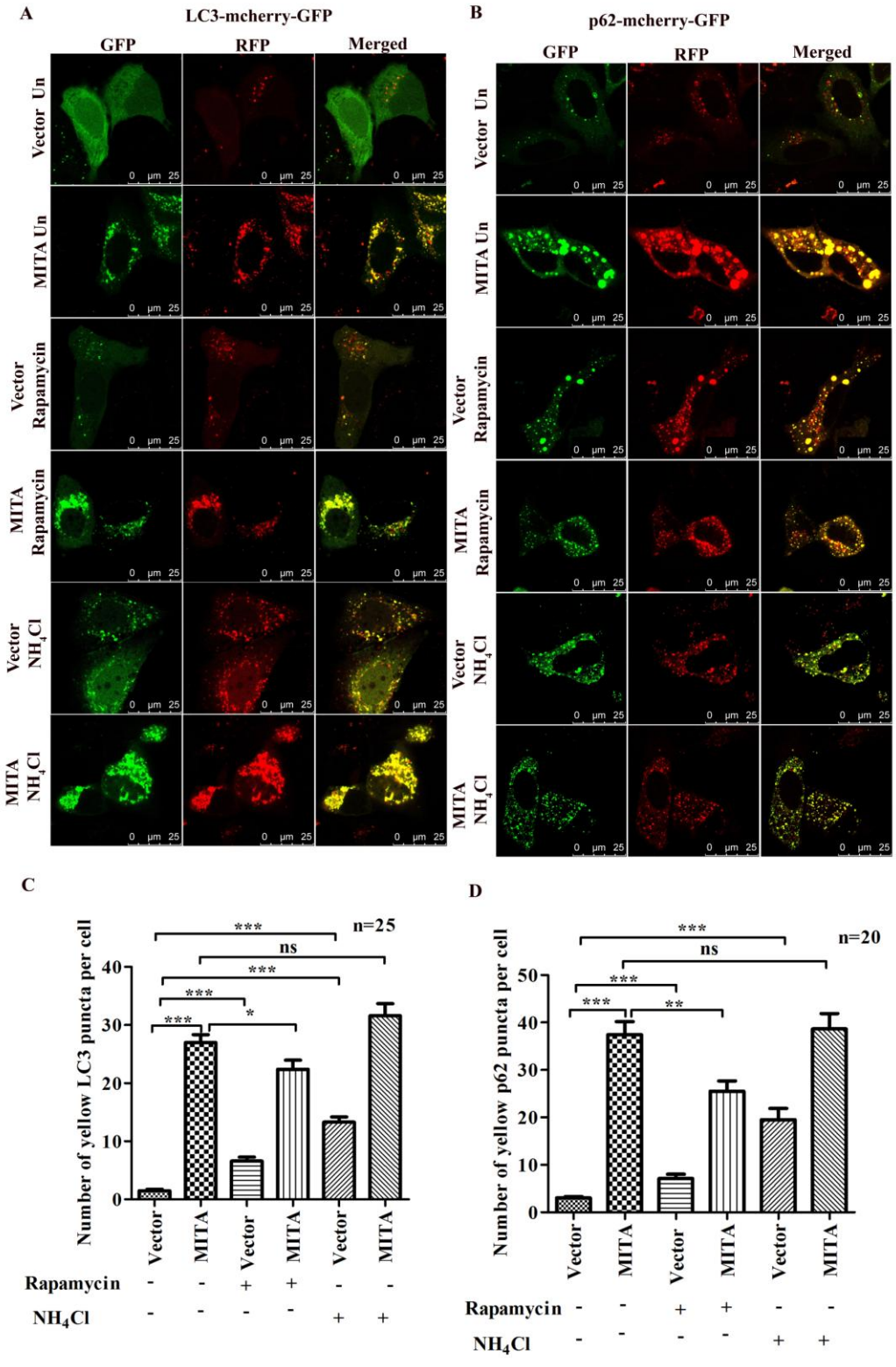

A
LC3-mcherry-GFP
GFP
RFP
Merged
Vector Un
MITA Un
Vector Rapamycin
MITA Rapamycin
Vector NH4Cl
MITA NH4Cl
B
p62-mcherry-GFP
C
Number of yellow LC3 puncta per cell
n=25
ns
Rapamycin
NH4Cl
D
Number of yellow p62 puncta per cell
n=20

Fig-4:

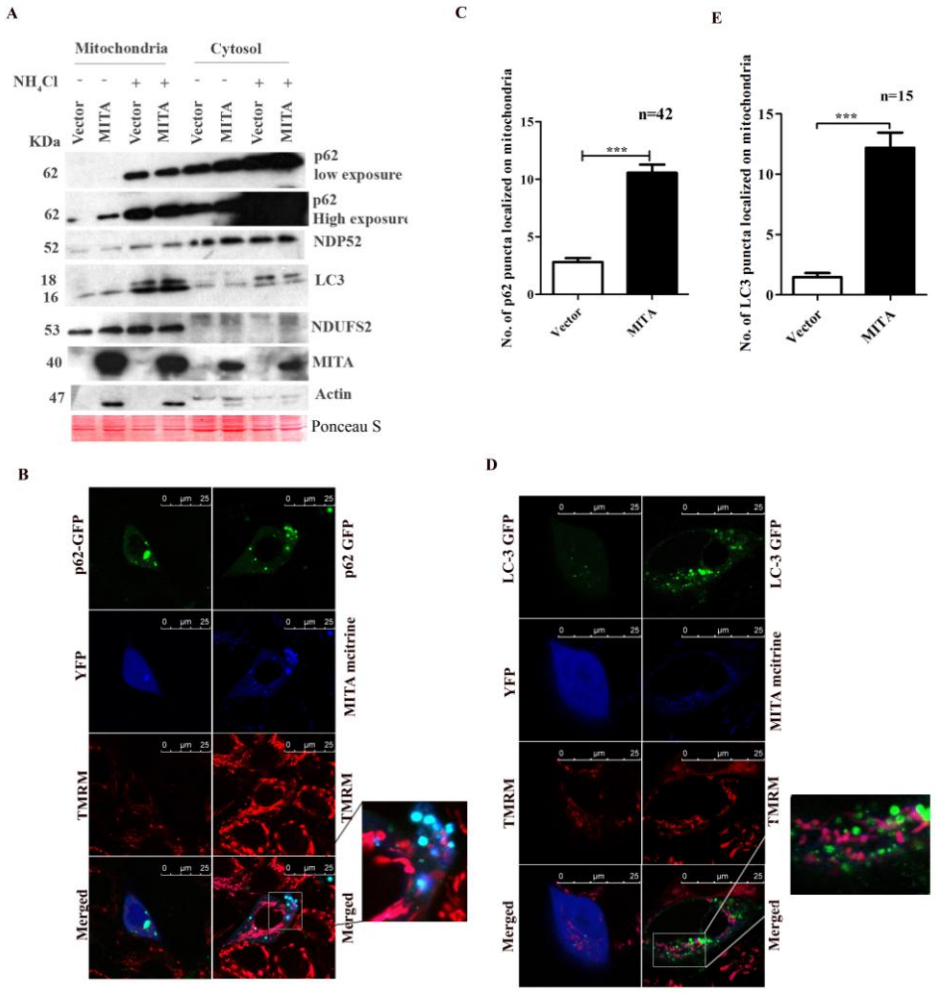
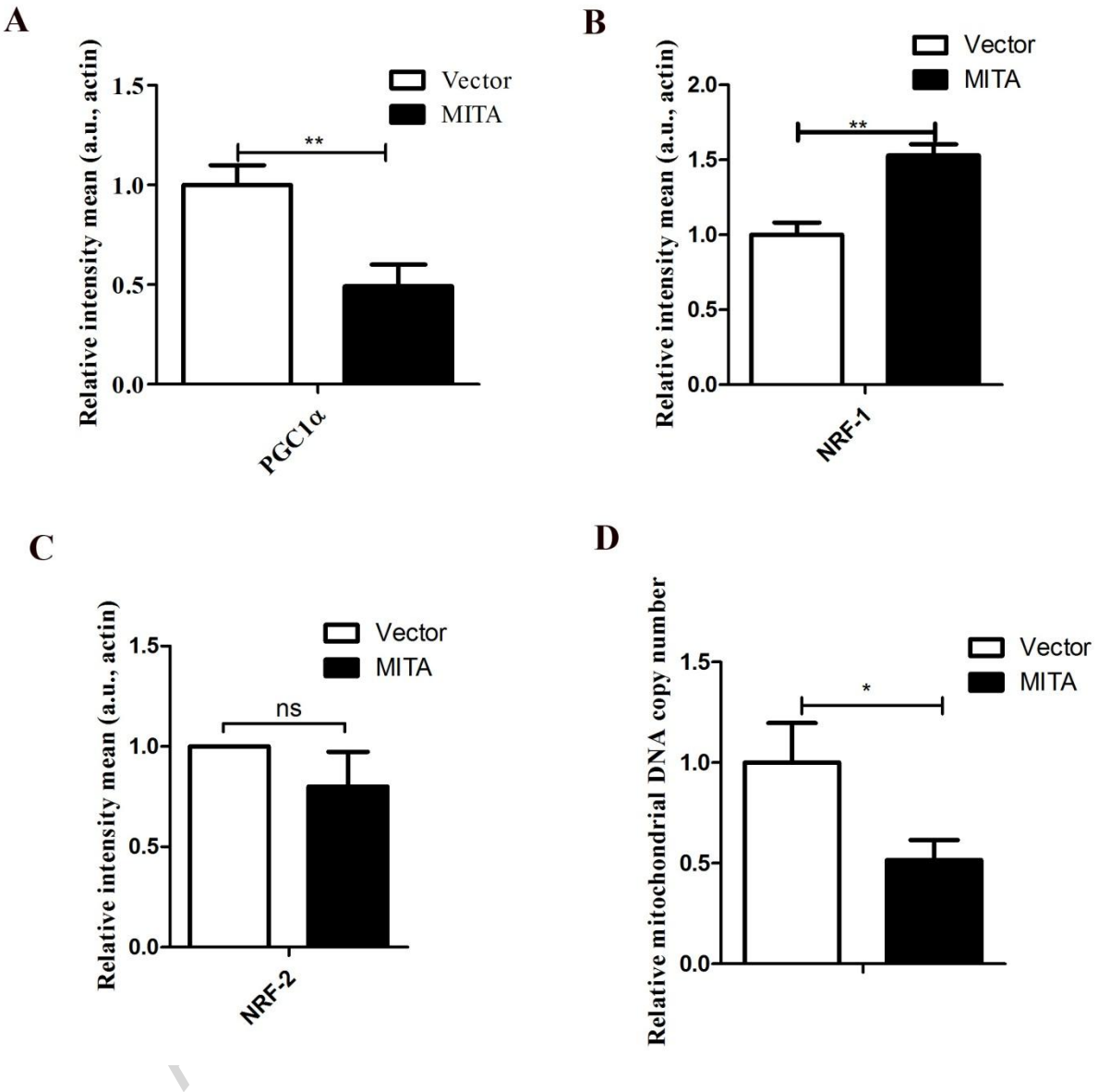


Fig-5:



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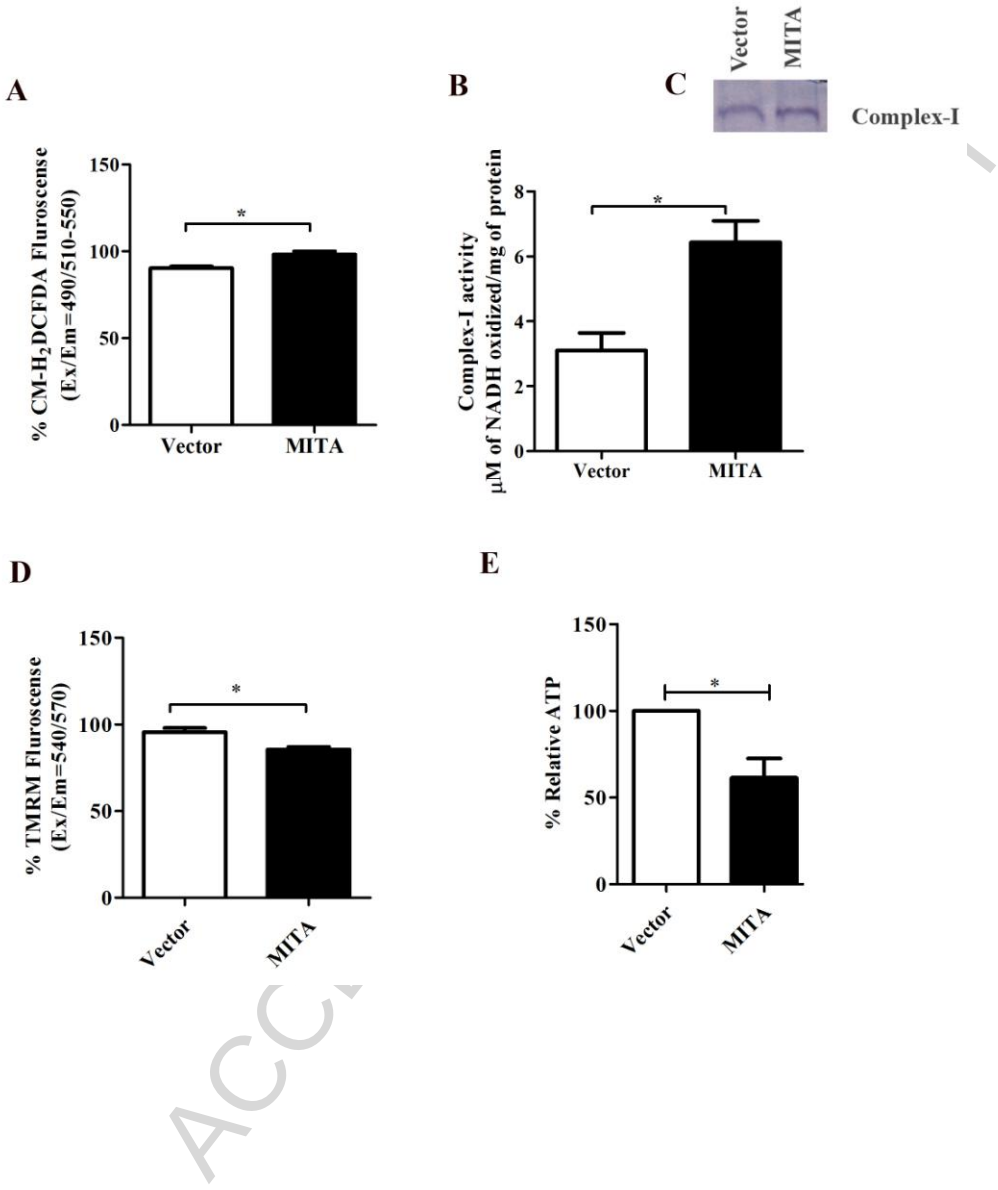
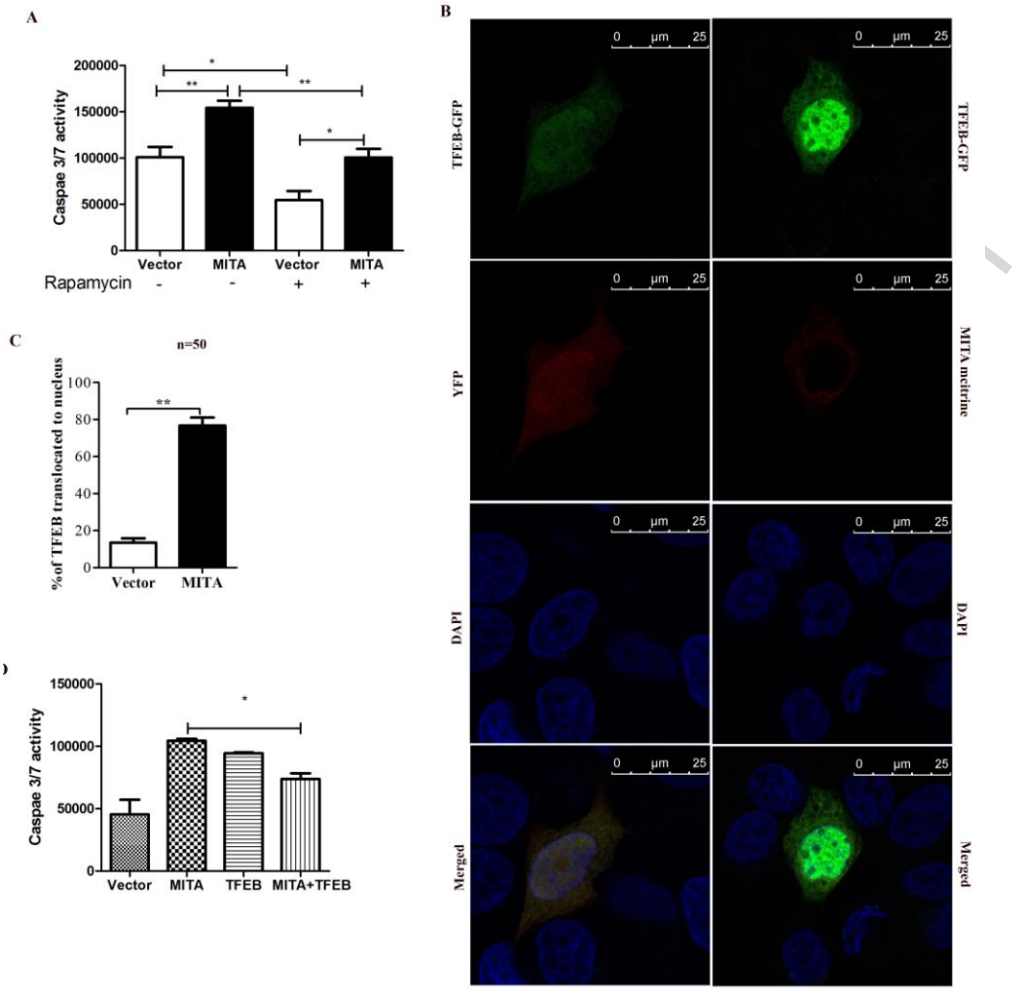
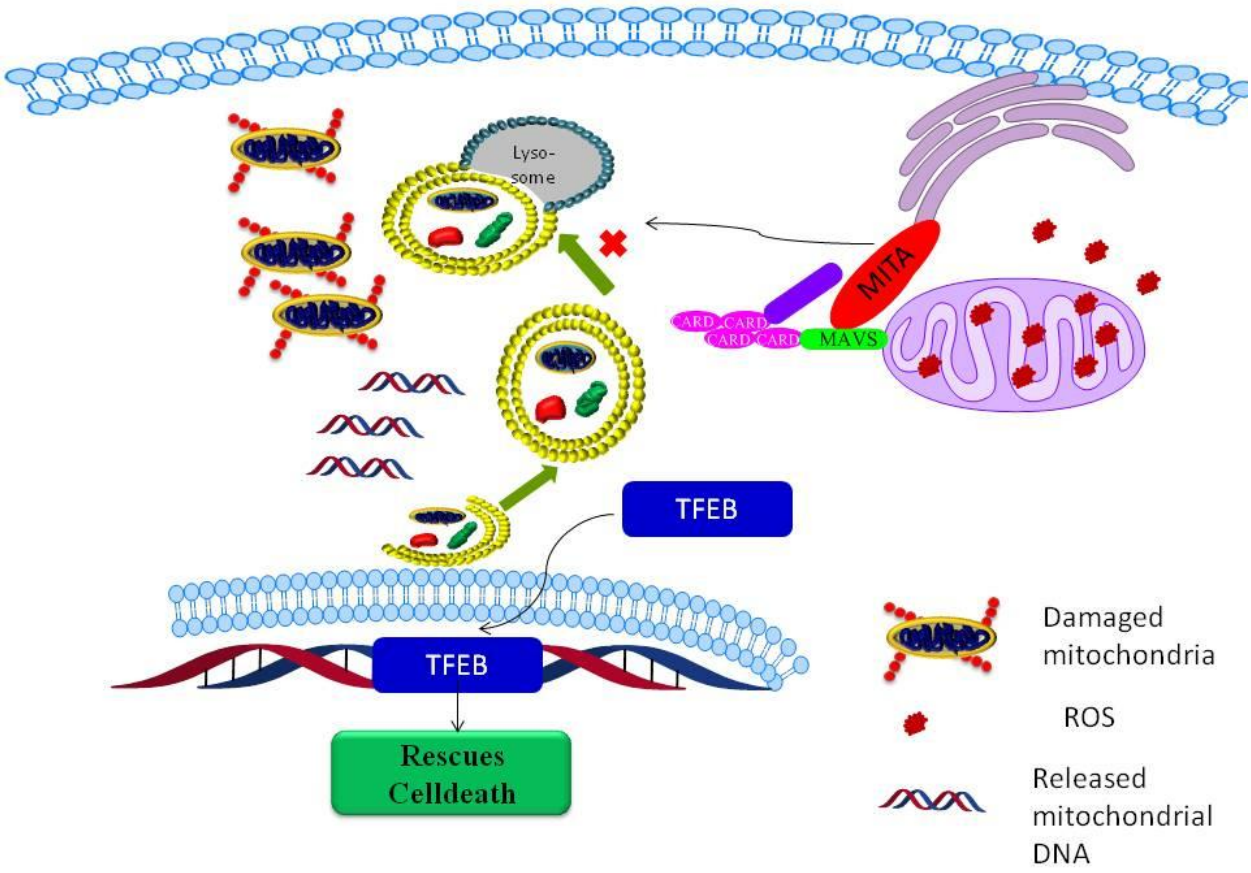


Fig-7:





Graphical abstract

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Antiviral signaling protein MITA acts as a tumor suppressor in breast cancer by regulating NF-κB induced cell death

Khyati Bhatelia [a], Aru Singh [b], Dhanendra Tomar [a], Kritarth Singh [a], Lakshmi Sripada [a], Megha Chagtoo [b], Paresh Prajapati [a], Rochika Singh [a], Madan M. Godbole [b], Rajesh Singh [c,*]

[a] Department of Cell Biology, School of Biological Sciences and Biotechnology, Indian Institute of Advanced Research, Gandhinagar, India
[b] Deptartment of Endocrinology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, UP, India
[c] Department of Bio-Chemistry, The M. S. University of Baroda, Vadodara 390005, Gujarat, India

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ABSTRACT

Emerging evidences suggest that chronic inflammation is one of the major causes of tumorigenesis. The role of inflammation in regulation of breast cancer progression is not well established. Recently Mediator of IRF3 Activation (MITA) protein has been identified that regulates NF-κB and IFN pathways. Role of MITA in the context of inflammation and cancer progression has not been investigated. In the current report, we studied the role of MITA in the regulation of cross talk between cell death and inflammation in breast cancer cells. The expression of MITA was significantly lower on in estrogen receptor (ER) positive breast cancer cells than ER negative cells. Similarly, it was significantly down regulated in tumor tissue as compared to the normal tissue. The overexpression of MITA in MCF-7 and T47D decreases the cell proliferation and increases the cell death by activation of caspases. MITA positively regulates NF-κB transcription factor, which is essential for MITA induced cell death. The activation of NF-κB induces TNF-α production which further sensitizes MITA induced cell death by activation of death receptor pathway through capsase-8. MITA expression decreases the colony forming units and migration ability of MCF-7 cells. Thus, our finding suggests that MITA acts as a tumor suppressor which is down regulated during tumorigenesis providing survival advantage to tumor cell.

1. Introduction

Breast cancer is the second most common form of cancer worldwide. About 1.3 million women are diagnosed with breast cancer annually and more than 4,000,000 women die from the disease around the world [1,2]. In spite of extensive efforts, there is significant morbidity and mortality associated; therefore, understanding the pathogenesis of breast cancer is of immense importance.

Evidences support the view that chronic inflammation contributes to initiation and progression of cancer [3–5]. The patients with ulcerative colitis and Crohn's disease are at increased risk for developing colorectal cancer. Similarly, inflammation and infection of liver are associated with increased risk of hepatic cancer [6,7]. The experimental evidences demonstrating association of inflammation and breast cancer are emerging. Chronic inflammation plays a critical role in breast cancer occurrence/recurrence [8]. Inflammatory Breast Cancer (IBC) is one of the most aggressive types of breast cancer. The symptoms of IBC like swelling, skin redness, and an orange peel like texture of the skin are similar to inflammation. IBC is often misdiagnosed as mastitis and even antibiotics are prescribed to the patients [9]. These observations suggest that there is a strong linkage between inflammation and breast cancer. The biochemical mechanisms regulating inflammation in breast tissue and their association with breast cancer are not understood.

NF-κB and IFNs are important cellular pathways associating inflammation and cancer. The regulation of NF-κB and IFN pathways is extensively studied; however, its modulation in stimulus specific manner and its significance to tumorigenesis are still not clear. Recent studies suggest that sub-cellular organelles, specifically mitochondria and ER, provide novel signaling platform for the assembly of signalosomes. Mitochondria are emerging as a central regulator of viruses and bacteria induced inflammatory pathways. The discovery of mitochondria associated viral signaling protein (MAVS) on the outer membrane of mitochondria and its role in regulating NF-κB and IFN pathway during viral and bacterial infections suggested a strong linkage between mitochondria and inflammation [10]. Similarly, ER associated protein MITA is another link that might help understand the linkage between ER, mitochondria and inflammation.

MITA plays an important role in inflammation through regulation of NF-κB and IFN [11]. MITA interacts with RIG-I, and MAVS associated signalosome. This further activates downstream kinase complexes: the 'non-canonical' IKK-related kinase TBK1 or IKK complex [12]. The TBK1 complex induces the phosphorylation and dimerization of the transcription factors (IRF3 and IRF7), which translocate to the nucleus

* Corresponding author at: Department of Bio-Chemistry, Faculty of Science, Lokamanya Tilak Road, Sayajigunj, Vadodara 390002, Gujarat, India. Tel.: +91 265 2759594.
E-mail address: singhraj1975@gmail.com (R. Singh).

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and bind to IFN-stimulated response elements (ISREs), thereby expressing type I IFN genes and downstream IFN-inducible genes [13]. On the other hand IKK complex activates NF-κB, subsequently promoting the expression of pro-inflammatory cytokines and other cell survival/death genes.

Given the strong linkages of inflammation and cancer, we hypothesize that MITA may be critical regulator of either cell survival or cell death, however, evidences are still lacking. We studied the expression of MITA in tumorous tissues of human breast cancer patients as well as in different breast cancer cell lines and investigated its role as a potential tumor suppressor. We observed that expression of MITA is predominant in extra-tumoral tissue whereas lower in tumorous tissue. MITA sensitizes the breast cancer cells to TNF-α induced cell death. MITA induced NF-κB is essential for cell death as well as clonogenic ability of the cells.

2. Materials and methods

2.1. Cells and cell culture

MCF7, T47D and HBL100 breast cancer cell lines were obtained from National Center for Cell Sciences, Pune, India. MDA-MB-231 was a gift of Prof. R. P. Singh (Central University of Gujarat, India). MCF-7, ZR-75-1 and T47D cells were cultured in RPMI 1640 (Life Technologies, USA), HBL100 in Dulbecco's modified Eagle's medium (Life Technologies, USA) and MDA-MB-231 in Leibovitz's L-15 media (HI-MEDIA, India). The media used were supplemented with 10% FBS (Life Technologies, USA) and 1% penicillin, streptomycin, and neomycin (PSN) antibiotic mixture (Life Technologies, USA). Cells were incubated at 37 °C, 5% CO2 in specified media. MCF 10A cells were cultured in DMEM F12 (INVITROGEN) base media supplemented with (10% horse serum) along with the following supplements: 1) cholera toxin (100 ng/ml), 2) EGF (20 ng/ml), 3) hydrocortisone (500 ng/ml), and 4) insulin (cell culture tested) (10 μg/ml).

2.2. Plasmids and reagents

MITA cloned in pCMV6-ENTRY plasmid was a gift from Dr. Hong-Bing Su (Wuhan University, China). p65shRNA (RelA1 shRNA and RelA2 shRNA) and control shRNA were provided by Dr. Edurne Berra Ramírez (Gene Silencing Platform, CICbioGUNE, Derio, Spain). MITA shRNA was a generous gift by Dr. Peter Chumakov (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences). FDEVDG$_2$ construct was gifted by Dr. Brian Seed (Department of Genetics, Harvard Medical School, Cambridge Street, Boston). Primary antibodies used were MITA (Proteintech, USA), caspase-8, PARP, p65 (Cell Signaling Technology, Inc., USA), β-Actin and GAPDH (Abcam, USA), IκBα (Cell Signaling Technology, Inc., USA). HRP-conjugated anti-rabbit and anti-mouse antibodies (Thermo Scientific, USA) were used. The reagents used were TNF-α (Tumor necrosis factor) (Biovision, USA), PDTC (Pyrrolidine dithiocarbamate) (Sigma Aldrich, USA) zVAD-fmk (N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone) (Biovision, USA), IETD-fmk (Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone) (Clontech, USA).

2.3. Transfection

MCF-7 cells were transfected using standard calcium phosphate method [14]. MCF-7 cells and T47D were transfected using X-treme gene transfect reagent (Roche, Germany). HBL100 cells were transfected with X-tremeGENE 9 DNA transfection reagent (Roche, Germany) as per manufacturer's protocol.

2.4. Collection of tissues

Human breast tumor specimens were obtained from patients undergoing surgery. Tissues were collected from the tumor zone (tissue within the tumor boundary), and normal zone (distal normal tissue at least 10 mm from the outer tumor boundary). A fraction of all tissues was fixed in formalin and embedded in paraffin for routine histopathological analysis. The rest of the fractions were frozen in liquid nitrogen and then stored at −80 °C for RNA and protein extraction. Ethical approval from institute's ethical committee was taken prior to collection of sample for each of the patients. Details of the tissue specimen used are given in Supplementary Table 1.

2.5. Immunohistochemistry

After de-paraffinization in xylene and hydration by gradient alcohol series, antigen retrieval was done by heat treatment in citrate buffer (10 mM, pH 6.0). The sections were incubated in 10% NSS (normal sheep serum) for 20 min to block non-specific binding and further incubated with antibodies against MITA (1:1000) in 0.1% BSA overnight at 4 °C. Sections were stained using Quick Universal ABC KIT (Vector) followed by peroxidase staining reaction with DAB/H_2O_2 as chromogen. The stained sections were observed under bright field light microscope (Nikon Eclipse 80i; Nikon Instech Co. Ltd., Kawasaki, Kanagawa).

2.6. Quantitative analysis of gene expression

Total RNA was isolated using Tri Reagent (Life technologies, USA) and was reverse transcribed to synthesize cDNA using Transcriptor First Strand cDNA synthesis kit (Roche, Germany) or SuperScript VILO cDNA Synthesis Kit (Life technologies, USA) according to the manufacturer's instructions. Real time PCR was performed using SYBR Premix Ex TaqTM (Takara, Japan) or SYBR mix (life technologies, USA) or Applied Biosystems as per manufacturer's instructions.

Specific primers of the genes are listed below.

1. MITA: Fwd 5′-CGCCTCATTGCCTACCAG-3′;
   Rev, 5′-ACATCGTGGAGGTACTGGG-3′;
2. TNF-α: Fwd 5′-CCCAGGGACCTCTCTCTAATCA-3′;
   Rev 5′-GCTACAGGCTTGTCACTCGG-3′;
3. β-Actin: Fwd 5′-TCGTGCGTGACATTAAGGGG-3′;
   Rev 5′-GTACTTGCGCTCAGGAGGAG-3′;
4. 16s rRNA: Fwd 5′-GAAACCAGACGAGCTACCTAAG-3′;
   Rev 5′-GCCTCTACCTATAAATCTTCCC-3′;
5. GAPDH: Fwd 5′-AGAAGGCTGGGGCTCATTTG-3′;
   Rev 5′AGGGGCCATCCACAGTCTTC 3′.

2.7. Western blot

Cells were plated at a density of 4.5 × 10^5 cells/well in the six well plate and transfected with indicated expression plasmid or shRNAs using calcium phosphate method. After 48 h of transfection, the cells were harvested, washed with ice cold PBS and lysed in buffer A (150 mM NaCl, 30 mM Tris–Cl, 10% Triton X-100, 10% Glycerol, 1× Protease Inhibitor (Roche, Germany). The equal protein was loaded and resolved on 11% SDS-PAGE. Protein was electroblotted on PVDF membrane at 110 V for 1 h at 4 °C. The membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS) or 5% BSA (BSA (Sigma-Aldrich, USA), 0.1% Tween-20 in TBS-0.02 M Tris–Cl, 0.15 M NaCl) for 1 h at room temperature. The membrane was incubated overnight with specific primary antibody. After incubation, the membrane was washed three times with TBS-T (TBS containing 0.1% Tween-20) for 10 min and incubated with a secondary antibody at room temperature for 1 h. The membrane was washed three times with TBS-T and signal was visualized by using EZ-ECL chemiluminescence detection kit for HRP (Biological Industries, Israel) by exposing it to X-ray film.

For the western blotting from tissue samples, the tissue samples obtained from breast cancer patient were snap frozen in liquid nitrogen. The tissue was homogenized to fine powder in the presence of liquid

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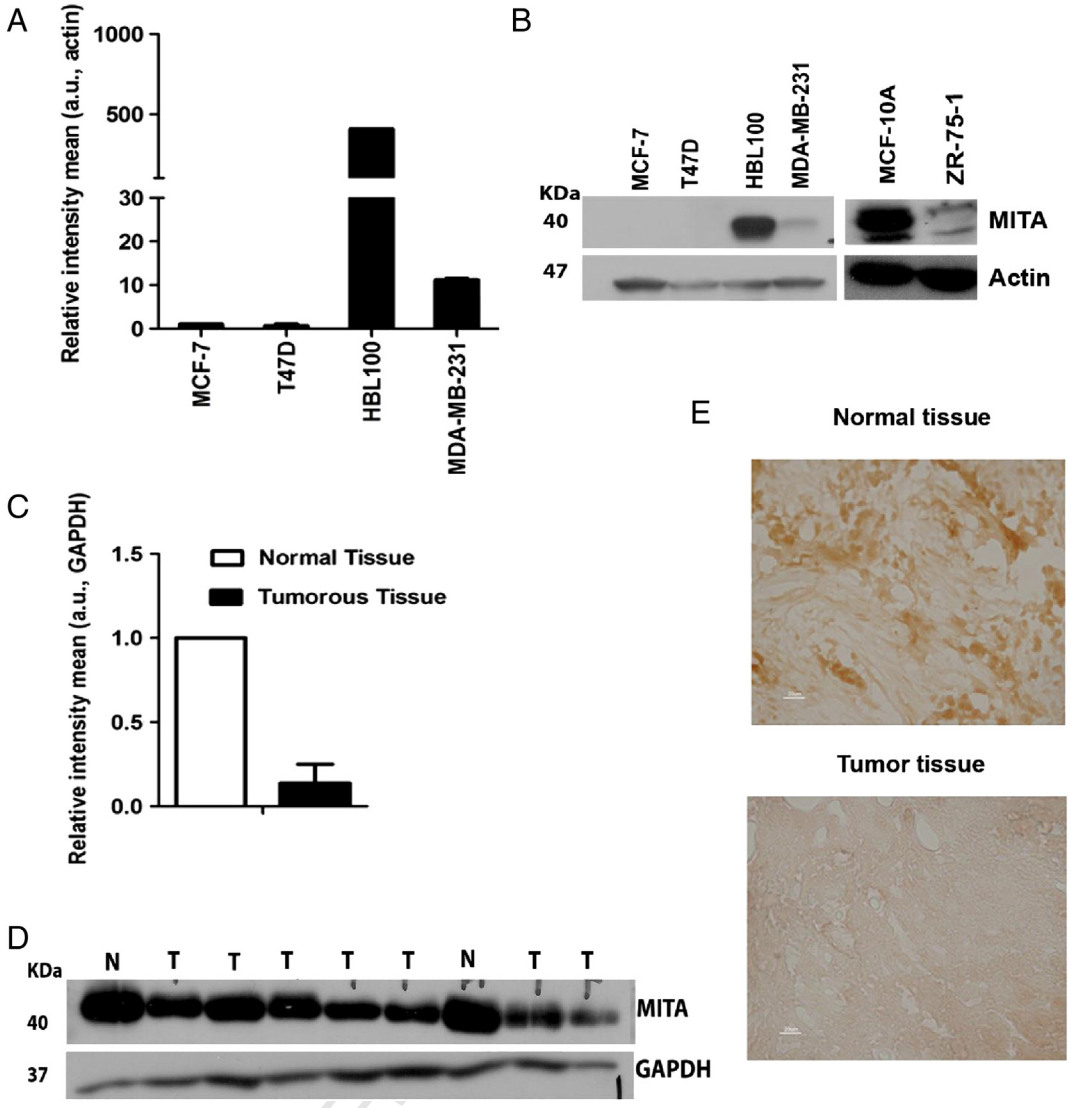

Fig. 1. Analysis of expression of MITA in breast cancer cell lines and different tumor tissue of breast cancer patient: (A) RNA was isolated from MCF7, T47D, HBL100 and MDAMB231 breast cancer cell lines, cDNA prepared and quantitative expression of MITA was analyzed using qPCR. (B) Protein level expression of MITA in different breast cancer cell lines was analyzed using western blot analysis using antibody against MITA. (C) RNA was isolated from tumorous and extra-tumoral tissues of breast cancer patients and relative expression of MITA was analyzed using qPCR. (D) Protein level expression of MITA was analyzed in the tumoral and extra tumoral tissue by western blot analysis using antibody against MITA. (E) Immunohistochemical analysis of tumoral and extra-tumoral tissues was done by incubating the tissue sections with antibody against MITA and detected using DAB staining.

nitrogen and lysed in RIPA lysis buffer(50 mM Tris [pH 7.4], 50 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.1% SDS, and 1% Triton X-100, 0.2% protease inhibitor cocktail, 1 mM PMSF, 2 mM NaF and 2.5 mM sodium pyrophosphate). The lysates were freeze thawed three times in liquid nitrogen. After 15 min of centrifugation (4000 rpm at 4 °C), the supernatant was saved to use as a whole-cell lysate. The protein was analyzed by western blotting as described above.

2.8. NF-κB luciferase assay

To assess NF-κB activity, MCF-7 cells were plated at density of 1×10^5 cells/well in 24 well plate and luciferase assay was performed as described previously using Dual-Glo luciferase assay system (Promega, USA) [15].

2.9. Caspase 3/7 and caspase-8 activity assay

The activity was performed using Caspase-Glo^R 3/7 Assay kit (Promega, USA) or Caspase-Glo^R 8 Assay kit (Promega, USA). Cells were plated at the density of 4×10^4 cells per well in 96 well white clear bottom plate and transfected with indicated expression plasmids or shRNAs and respective controls. Caspase-Glo^R 3/7 (10 μl) reagent or caspase-8 Glo reagent was added to each well and luminescence was measured with a Centro LB 960 Luminometer (Berthold Technologies, Germany).

2.10. Secreted GLUC activity assay for caspase activation in culture supernatant

Cells were plated in 24 well plate and co-transfected with MITA and a reporter construct FDEVDG2 [16]. The construct has a DEVD site placed in between GLUC reporter (*Gaussia* luciferase) and β-actin, so once the substrate site is cleaved by the caspases, luciferase will be secreted in the supernatant. After 24 h of transfection, the cells were treated with specific inducer of cell death. The supernatant (SN) was collected and centrifuged at 14,000 rpm for 5 min. Supernatant was diluted in 1:10 in 100 μl 1× lysis buffer. The substrate was added. 50 μl of substrate was added to 10 μl of this mixture and was analyzed with Centro LB 960 Luminometer (Berthold Technologies, Germany). Attached cells were lysed in 1× lysis buffer 100 μl per well for 15 min.

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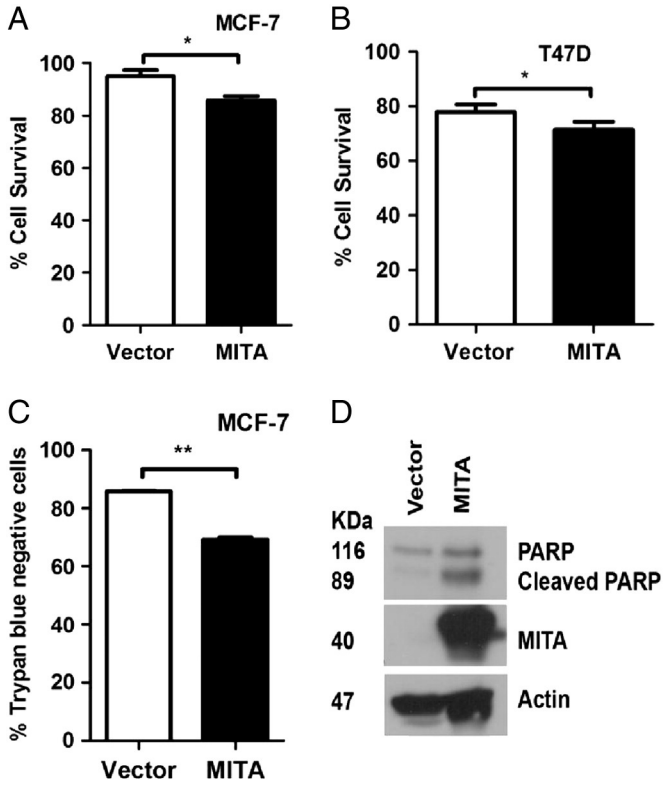

Fig. 2. MITA induces cell death: The specified cells were transfected with MITA and cell survival was monitored using (A and B) MTT reduction assay. Cell death was measured in MCF-7 cells by (C) trypan blue exclusion assay and (D) PARP cleavage.

10 μl of lysate was added to 50 μl of 1× substrate to detect that cellular GLUC activity luminescence was measured with a Luminometer. Total caspase activity was calculated in the SN as well as in cell lysate, and total caspase activity was calculated as the ratio of the caspase activity in SN versus (vs) cellular caspase activity.

2.11. Trypan blue exclusion assay

Cells were plated at the density of 1×10^5 cells/well in 24 well plate and transfected with the specific constructs. After 24 h of transfection, the cells were treated with TNF-α (10 ng/ml) for 24 h and stained with trypan blue. Minimum 100 cells per view were counted and percentage of cell survival was plotted.

2.12. MTT assay

The cellular proliferation was analyzed by MTT assay. MCF7 cells were plated in 24-well plate at a density of 1×10^5 cells/well. The cells were transfected with MITA as well as vector. After 24 h of transfection, 20 μl of MTT solution (5 mg/ml) (Serva, Germany) was added to each well and incubated for 2 h. After incubation, 500 μl of solubilization buffer (2% w/v SDS, 18.5% w/v formaldehyde) was added to dissolve the precipitate of purple colored formazan and color intensity was monitored using colorimetric microplate reader (BioTek Instruments, Inc. USA) at 595 nm wavelength.

2.13. Colony formation assay and scratch assay

Clonogenic activity of cells and migration ability of cells were determined as described previously [15,17].

2.14. Statistical analysis

Data are shown as mean ± SEM for no. of times experiment was repeated. Comparisons of groups were performed using student t-test for repeated measurements to determine the levels of significance for each group. The experiments were performed minimum two times independently and $p < 0.05$ was considered as statistically significant. GraphPad Prism was used to perform all the statistical analysis.

3. Results

3.1. Expression analysis of MITA in different breast cancer cell lines and patient samples

To study the role of MITA in initiation and progression of breast cancer, we analyzed the expression of MITA in different breast cancer cell lines. Relative expression of MITA in four different breast cancer cell lines (MCF-7, T47-D, HBL100 and MDA-MB-231) was analyzed by quantitative Real Time PCR. The expression of MITA was significantly lower in MCF-7 and T47-D cell lines as compared to HBL100 and MDA-MB-231 cells (Fig. 1A). The protein levels of MITA were checked in the same set of cell lines as well as in MCF-10A (non-tumorigenic mammary epithelial cells) and ZR-75-1 (ER-positive) cell lines by western blotting. The intense band of 40 kDa band corresponding to MITA was observed in HBL-100 indicating the strong expression of MITA. Similarly the non-tumorigenic cell line MCF-10A showed high level of MITA expression (Fig. 1B). The lower level of MITA was also observed in MDA-MB-231 as compared to HBL100 whereas it remained undetected in MCF7 and T47D (Fig. 1B). Similarly, ER positive cell line ZR-75-1 showed low level expression of MITA.

Expression of MITA was further investigated in tumor tissues obtained from breast cancer patients using quantitative real time PCR. Interestingly, significantly low RNA levels of MITA were observed in all tumorous tissues as compared to the extra-tumoral tissue (Fig. 1C). Similarly, protein levels of MITA were also low in all tumorous tissue as compared to the extra-tumoral tissue of the same patient (Fig. 1D). The expression of MITA was also analyzed by immunohistochemistry. Intense staining of MITA observed in case of extra-tumoral tissue as compared to tumorous tissue confirmed our observations (Fig. 1E). These evidences suggest that MITA is primarily expressed at higher levels in extra-tumoral tissue and pre-malignant cell lines, whereas, it decreases significantly in tumorous tissue and malignant breast cancer cell lines.

3.2. MITA induces cell death in breast cancer cell lines

As the expression of MITA decreased in tumorous tissue from breast cancer patient as well as in malignant cell lines, we hypothesized that MITA may be a potential tumor suppressor either by regulating cell survival or cell death. MCF-7 cell line having relatively low expression of MITA was chosen for further experiments. MITA was overexpressed in MCF-7 and cell proliferation was monitored using MTT. The transfection of MITA in MCF-7 showed decreased cell survival as compared to vector transfected cells (Fig. 2A). To eliminate the cell line specific action, T47D cells were transfected with MITA and cell survival was monitored. Decrease in MTT reduction was observed in case of MITA expressing cells as compared to control indicating decrease in the cell survival (Fig. 2B).

To further confirm if MITA induced decreased proliferation is due to cell death, the effect of MITA expression on induction of cell death was analyzed by trypan blue exclusion assay. The expression of MITA in MCF-7 significantly decreased trypan blue negative cells as compared to control cells, indicating increased cell death (Fig. 2C). The mechanism of cell death was further investigated. PARP is an established marker of apoptosis as it is a target of executioner caspases and is cleaved during apoptosis [18,19]. MCF-7 cells were transfected with MITA and PARP cleavage was monitored after 24 h of transfection. The western blotting

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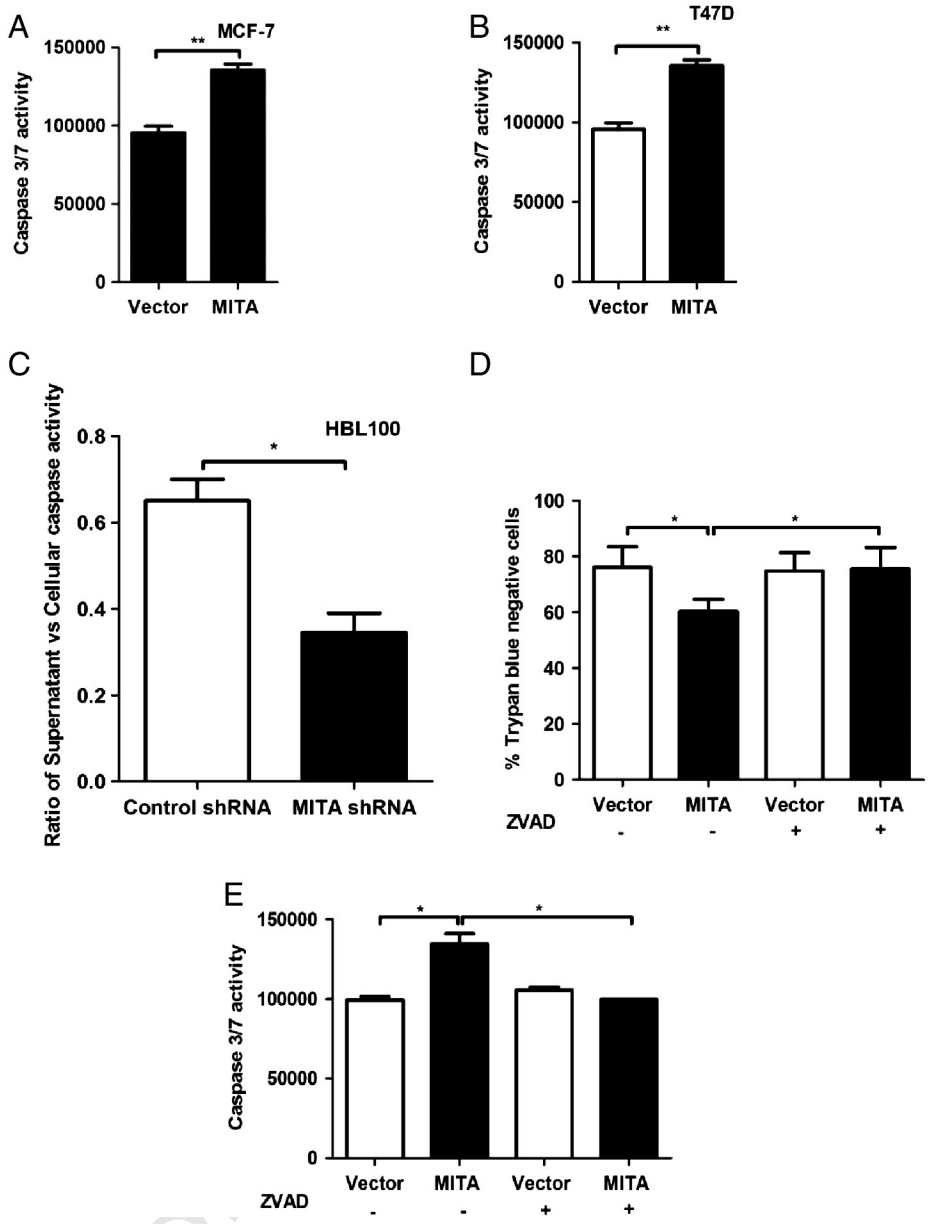

Fig. 3. MITA induces caspase activation during cell death: (A and B) The specified cells were transfected with MITA and caspase activity was measured using caspase glo substrate followed by luminescence measurement. (C) HBL100 cells were transfected with MITA shRNA along with the FDEVDG2 construct and the luminescence was measured in the SN and the cell lysate. Total caspase activity was calculated as the ratio of caspase activity in the SN vs cell lysate. (D and E) Caspases were inhibited in MITA transfected cells using PAN caspase inhibitor zVAD-fmk (20 μM) for 24 h. (D) Cell death was monitored using trypan blue exclusion assay and (E) caspase activity was measured using caspase glo substrate.

showed 110 kDa and 89 kDa band corresponding to native and cleaved form respectively. The expression of MITA showed increased levels of cleaved PARP (89 kDa) as compared to control (Fig. 2D).

The cleavage of PARP strongly suggests the activation of caspases in the presence of MITA. Caspases play a major role in initiation and execution of cell death; hence caspase 3/7 activation was analyzed using luciferase assay. The expression of MITA in MCF-7 significantly increased luminescence indicating enhanced caspase3/7 activity (Fig. 3A). The caspase activity was also monitored in T47D cell line in the presence of MITA and similar results were observed (Fig. 3B). As MITA was observed at higher level in ER negative HBL100 cells, MITA was knocked down in these cells to further confirm the role of MITA in cell death. The total caspase activity was monitored in HBL100 cell line using Gluc reporter based luciferase assay system in the MITA knocked down condition. The basal caspase activity decreased significantly upon MITA knocked down in HBL100 cells (Fig. 3C). The result further strengthened our hypothesis that MITA induces cell death by activating caspases. The role of caspases in cell death was further validated by inhibiting caspases using pan caspase inhibitor zVAD-fmk and then monitoring the cell death. The treatment of MITA transfected MCF-7 cells by zVAD-fmk significantly increased trypan blue negative cells as compared to control (Fig. 3D). Caspase activity was monitored by luciferase assay to confirm the inhibition of caspases. Decrease in caspase activities was observed in the cells treated with zVAD-fmk (Fig. 3E). These observations strongly suggest that inhibition of caspases rescues MITA induced cell death.

3.3. MITA regulates cell death by inducing NF-κB

NF-κB is a key regulator of pro- and anti-apoptotic genes during cell death. MITA is a key regulator of central inflammatory pathway [11], hence the role of NF-κB was analyzed in MITA induced cell death. MITA was co-transfected with NF-κB luciferase reporter construct in MCF-7 cells and luciferase activity was measured. The significant

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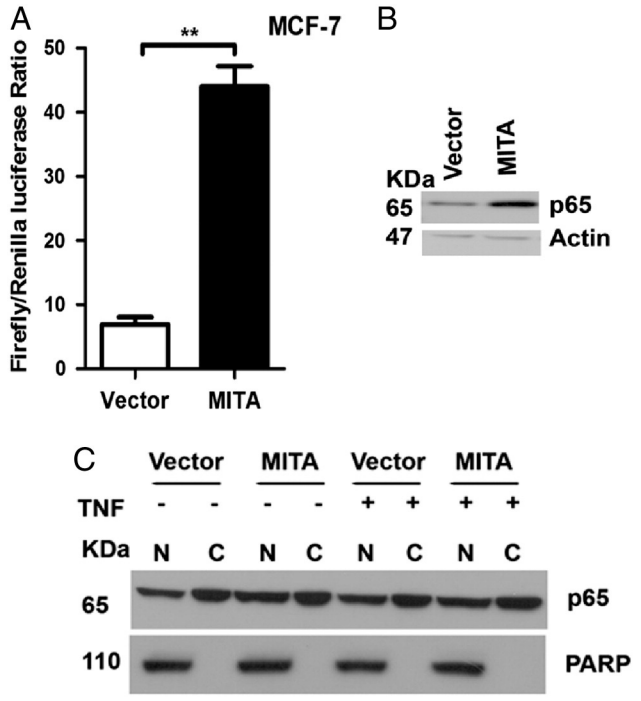

Fig. 4. MITA activates NF-κB transcription factor: (A) MITA was transfected in MCF-7 cells and NF-κB activity was measured using luciferase assay system. The activity was normalized and represented as Firefly/Renilla ratio. (B) MITA was transfected in MCF-7 cells and the level of p65 was analyzed by western blot using anti-p65 antibody. (C) Nuclear fraction and cytosolic fraction of MITA transfected cells were subjected to western blot analysis using p65 specific antibody.

increase in luciferase activity was observed in MITA transfected cells as compared to control indicating activation of NF-κB by MITA (Fig. 4A). 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 [20]. Therefore we analyzed the expression of p65 in the presence of MITA by western blotting. Elevated level of p65 was observed in MITA expressing cells as compared to control (Fig. 4B). During NF-κB activation, p65/p50 translocates to the nucleus to execute its action. Therefore the translocation of NF-κB to the nucleus was analyzed by sub-cellular fractionation of MITA transfected cells along with the control. TNF-α was taken as a positive stimulus. We observed increase in the level of p65 in nuclear fraction of MITA transfected cells as compared to the vector in untreated as well as TNF condition (Fig. 4C). IKK complex is the central kinase complex during NF-κB activation [20]. Hence, to further understand the mechanism of NF-κB activation through MITA, components of IKK complex (IKKα, IKKβ, IKKγ) were knocked down using respective shRNAs in MITA transfected MCF-7 cells and NF-κB activity was measured using luminescence. p65, the most downstream component of NF-κB pathway was also knocked down using specific shRNA to analyze if MITA acts downstream of IKK complex. NF-κB activity was significantly suppressed upon any of the three components of IKK complex analyzed (Supplementary Fig. 1). The experimental evidences strongly suggest that MITA acts at IKK complex and activates NF-κB.

To understand the role of NF-κB activation in MITA induced cell death, NF-κB activation was inhibited using p65 shRNA as well as chemical inhibitor PDTC and cell death was monitored using trypan blue exclusion assay. The knockdown of p65 in MCF-7 significantly increased trypan blue negative cells in the presence of MITA as compared to control (Fig. 5A). Similar results were observed in case of chemical inhibition of NF-κB in MITA transfected cells (Fig. 5B). These evidences suggest that NF-κB activation is essential for cell death. We also confirmed the role of NF-κB in caspase activation by luciferase assay system. Increased caspase activity was observed in the presence of MITA; whereas knockdown of p65 using shRNA reverted back to the control (Fig. 5C). Similarly, the expression of MITA in MCF-7 cells treated with PDTC also showed no increase in caspase activity as compared to control (Fig. 5D). PARP cleavage was also monitored in similar conditions. The transfection of MITA showed increased levels of cleaved PARP (89 kDa) as compared to control. The knockdown of p65 in the presence of MITA showed decreased level of cleaved PARP as compared to control (Fig. 5E). These results convincingly demonstrate that MITA induced NF-κB is essential for the induction of cell death.

3.4. NF-κB regulates cell death by increasing TNF-α production

To further investigate the role of NF-κB in the regulation of MITA induced cell death, the expression of NF-κB regulated genes (BCL-XL, BCL-2, Bax, TNF-α, cIAP1, cIAP2 and XIAP) was screened in MITA transfected cells using quantitative PCR. Elevated expression of TNF-α was observed in the cells expressing MITA as compared to control (Fig. 6A); however no significant difference was observed in the expression of other genes (data not shown). The role of increased TNF-α and its contribution in regulation of MITA induced apoptosis were further investigated. MITA transfected cells were treated with TNF-α for 24 h and cell death was monitored. Significant decrease in the percentage of trypan blue negative cells was observed in TNF-α treated cells as compared to untreated cells in the presence of MITA indicating sensitization of MITA induced cell death by TNF-α (Fig. 6B).

Caspase-8 is a key player of TNF induced cell death therefore it was hypothesized earlier that caspase-8 activation may be an important regulator of cell death induced by MITA [21]. MCF-7 cells were transfected with MITA and caspase activation was monitored by western blotting. An intense band of 43/41 kDa corresponding to cleaved caspase-8 was clearly observed in MITA transfected cells as compared to control (Fig. 6C). To further confirm the role of caspase-8 in MITA induced cell death, caspase-8 was inhibited using a specific inhibitor IETD-fmk and cell death was monitored. Inhibition of caspase-8 increased the number of trypan blue negative cells in MITA expressing cells as compared to control (Fig. 6D). HBL100 cells showed high expression of MITA; hence we monitored the effect of knockdown of MITA on caspase-8 activity. The knockdown of MITA in HBL100 showed decreased caspase-8 activity (Fig. 6E). This indicates that caspase-8 plays a key role in MITA induced cell death. The above results showed that MITA expression activates NF-κB and induces the expression of TNF and that may initiate death receptor pathway. To confirm this p65 was downregulated by p65 shRNA in MCF-7 cells in the presence of MITA and caspase-8 activity was monitored. The caspase-8 activity was observed to be equivalent to control which was otherwise increased in case of MITA transfected MCF-7 cells (Fig. 6F). These evidences strongly suggest that endogenous expression of MITA may sensitize breast cancer cells to TNF induced cell death and its loss in tumor cells provided survival advantage.

3.5. MITA decreases clonogenic ability of MCF-7 cells

The experimental evidences in the current study showed that MITA is expressed at lower levels in breast tumor than normal cells. It also sensitizes MCF-7 to cell death. Therefore we hypothesized that it may be a potential tumor suppressor. This observation was confirmed by monitoring the clonogenic ability of the MFC-7 cells in the presence of MITA. The expression of MITA in MCF-7 cells significantly decreased colony forming units as compared to control (Fig. 7A). As we observed here that MITA induced NF-κB is responsible for cell death, the role of NF-κB in MITA induced reduction in clonogenic ability of the cells was also analyzed. MCF-7 cells were transfected with MITA along with control and p65 shRNA and clonogenic ability of the cells was monitored. The clonogenic ability of the cells significantly increased upon p65 knockdown in the presence of MITA as compared to control (Fig. 7B). These evidences strongly suggest that MITA decreases the clonogenic ability of MCF-7 cells by positively regulating NF-κB. We

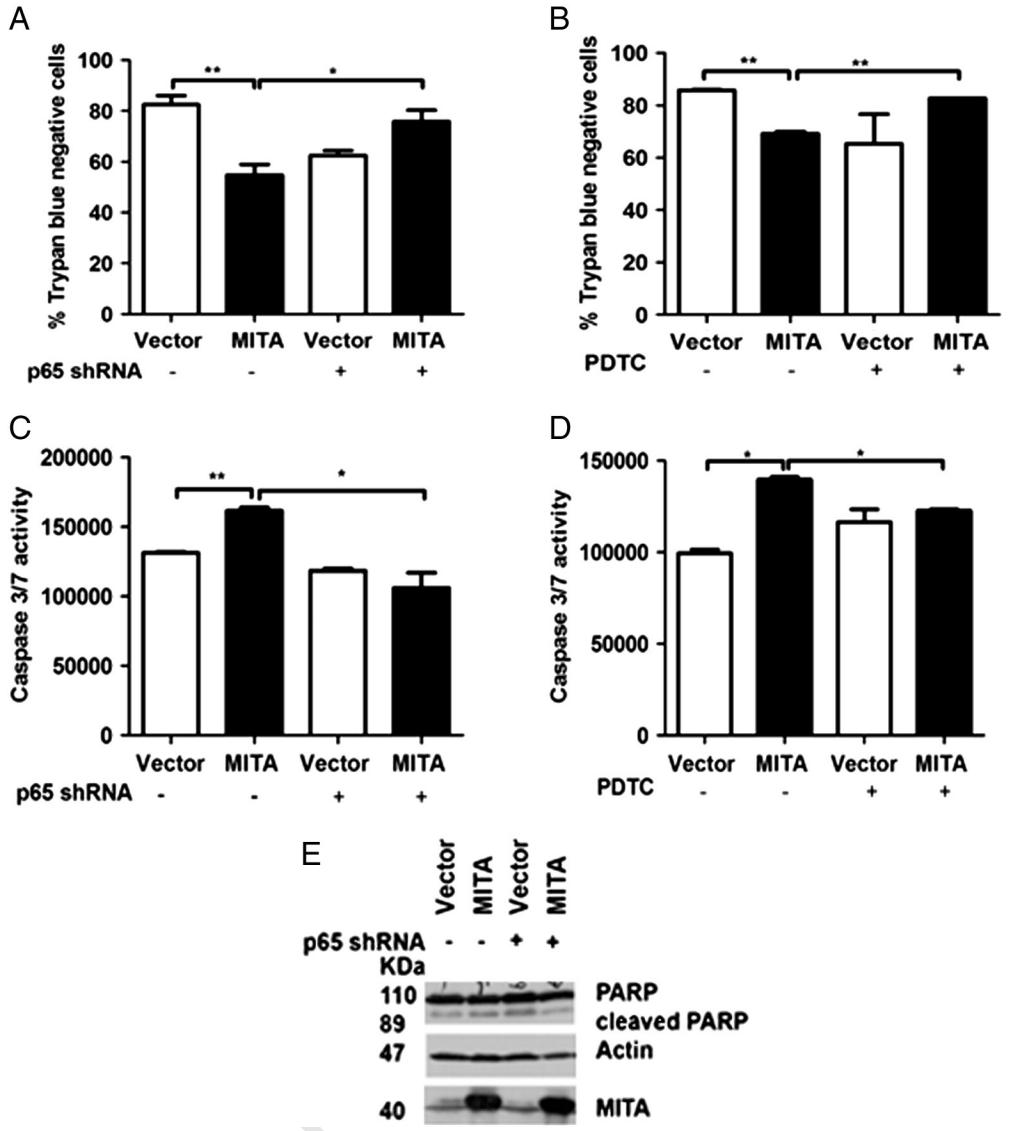

Fig. 5. MITA induced NF-κB is essential for cell death: MITA was transfected in MCF7 cells and NF-κB was inhibited using p65 shRNA or chemical inhibitor PDTC (100 μM) and cell death was quantified by (A and B) trypan blue exclusion assay. Caspase activity (C and D) and PARP cleavage were (E) also monitored in similar experimental conditions.

further checked the migration ability of MCF-7 cells. The cells were transfected with MITA and migration ability was analyzed using scratch assay. There was a significant increase in the open wound area of MITA transfected cells observed as compared to the control (Fig. 7C). To further confirm the role of MITA on migration ability of breast cancer cells, MITA was knocked down in HBL100 cells and its migration ability was monitored. There was a significant decrease observed in HBL100 cells upon MITA knock down (Fig. 7D). These observations strongly suggest that MITA regulated NF-kB negatively regulates clonogenic and migration ability of the breast cancer cells.

4. Discussion

The relation between inflammation and breast cancer is an emerging area. The current study is focused on ER resident MITA which is known to be essential for innate immune response against dsDNA virus and regulate both IFN and NF-κB pathway [11,22,23]. It has been observed that circulating tumor DNA levels increase in body fluids in different cancers including breast cancer [24–26]. Interestingly it had been observed that tumor DNA in complex with endogenous antimicrobial peptide LL37 can be transported back into endosomal compartments of plasmacytoid dendritic cells (pDC) leading to activation of type I IFNs [27]. It is possible that dsDNA induced pathway regulated by MITA may be linked to breast tumorigenesis which has not been investigated. In the current study, we demonstrated that MITA may be a potential tumor suppressor regulating NF-κB induced cell death in breast cancer.

The evidences in the current study suggest that expression of MITA is down regulated in tumor tissue as compared to normal. MITA is highly expressed in non-tumorigenic MCF10A cells whereas it is expressed at low levels in ER positive cells MCF-7, T47D and ZR-75-1. These findings suggest that during breast cancer progression, ER positive tumors specifically down regulate the proteins involved in innate immune response suggesting the evolved mechanisms to evade innate immune response pathways to facilitate tumor growth. This is further supported by loss of expression of RIG1, intracellular sensor of dsRNA, in ER positive cell lines [28]. It would be interesting to further study the correlation between the ER status and expression of MITA and other proteins involved in innate immune response and relevance during breast tumorigenesis.

The evidences in the current study clearly showed that MITA expression leads to cell death in breast cancer cell lines. The down regulation of MITA in tumor tissue and ER positive cell lines strongly suggests that it

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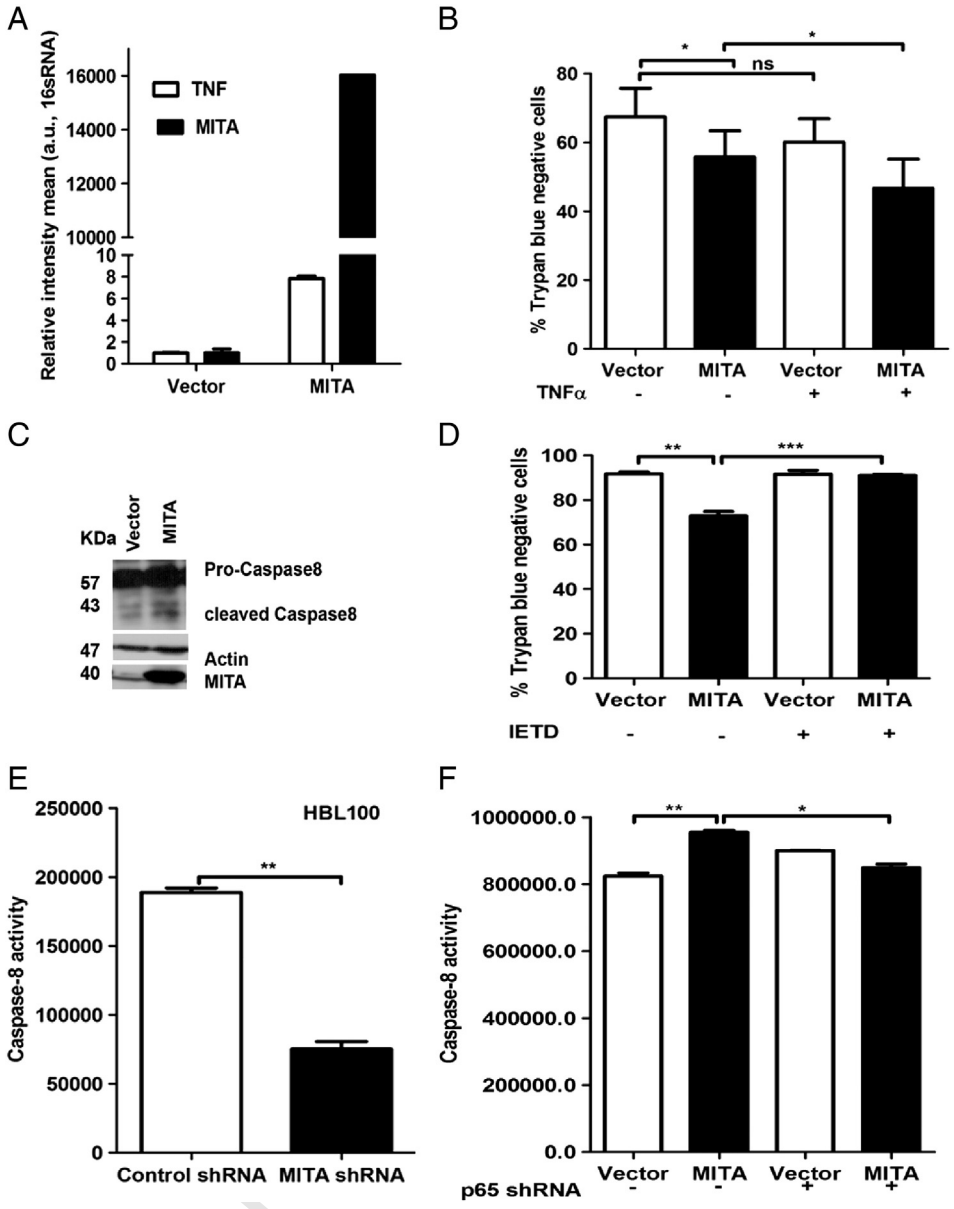

Fig. 6. MITA induced NF-κB sensitizes MCF-7 to TNF induced cell death: (A) MCF-7 cells were transfected with MITA. RNA was isolated and reverse transcribed to prepare cDNA. Relative mRNA level of TNF-α was quantified using real time PCR. (B) MITA transfected cells were treated with 10 ng/ml of TNF-α for 24 h and cell death was measured using trypan blue exclusion assay. (C) MCF-7 cells were transfected with MITA and caspase-8 activity was analyzed by using western blotting. (D) Caspase-8 activity was inhibited using IETD-fmk (1 μM) and cell death was monitored using trypan blue exclusion assay. (E) HBL100 cells were transfected with MITA shRNA and control random shRNA and caspase-8 activity was measured using caspase-8 glo substrate followed by luminescence measurement. (F) MITA was transfected in MCF7 cells and NF-κB was inhibited using p65 shRNA and caspase-8 activity was measured using luminescence.

may have important implication in regulating the cross talk of inflammatory and cell death pathway. As mentioned earlier, MITA is a critical regulator of NF-κB and IFN. These pathways are important cellular pathways associating inflammation and cancer [29,30]. The evidences here clearly demonstrated that MITA up regulates NF-κB pathway through IKK complex. Increased NF-κB activity has been found in both ER positive and ER negative breast cancer patients. The dysregulation of NF-κB and its implication to the breast cancer or any other cancer may be dependent on either loss or amplification of tumor suppressor or oncogene. The decreased expression of MITA and increased NF-κB activity provide advantage to the tumor cells. The association of NF-κB and breast cancer is further emphasized by recent observation of amplification of IKKε, a kinase regulating NF-κB pathway, in tumor tissue of breast cancer patients and breast cancer cell lines [31]. The gene is over expressed in over 30% of the breast carcinomas and provides survival advantage to tumor cells [31,32].

NF-κB is a dynamic transcription factor that induces the expression of several pro-apoptotic and anti-apoptotic genes. The role of NF-κB has been controversial as it may have pro-survival or apoptotic effect depending upon the stimulus and loss/gain of potential tumor suppressor/oncogene [33,34]. The current study also showed that MITA induced up regulation of NF-κB leads to high levels of TNF-α in breast cancer cells. Interestingly, TNF-α treatment further sensitized breast cancer cells to MITA induced cell death. TNF-α is known to bind to its receptor TNFR-I/II, either leading to NF-κB activation or cell death [35]. The p65 knockdown decreases the caspase-8 activity in MITA overexpressed conditions. The study strongly suggests that MITA induced NF-kB and increased level of TNF-α in breast cancer cells (MCF-7) lead to the activation of caspase-8 and downstream proteolytic cascade leading to cell death. The current study suggests that TNF-α shows antitumoric effect in the presence of MITA among its ability to play diverse role as pro or antitumoric agent. TNF in combination with melphalan is strongly

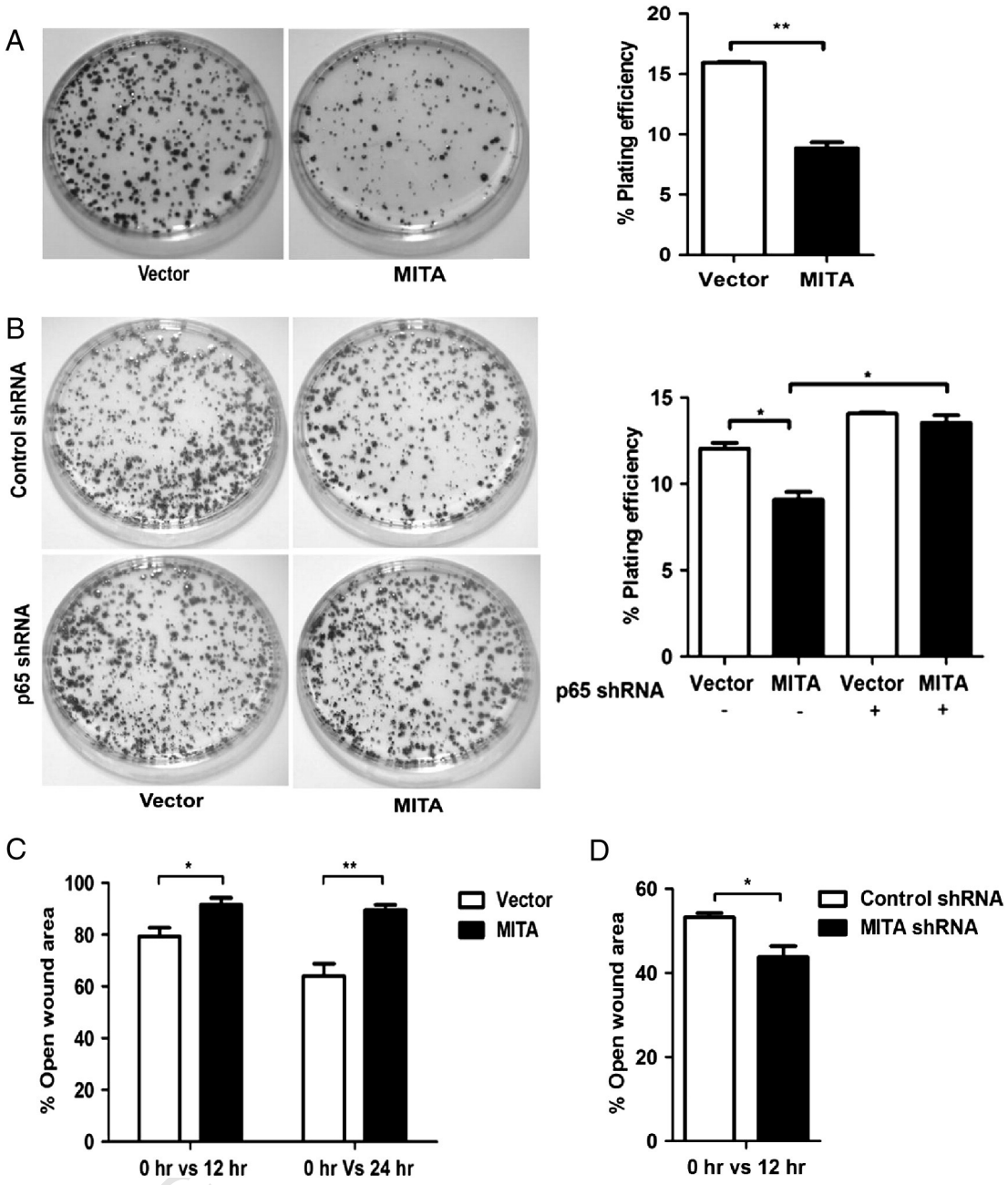

Fig. 7. The expression of MITA decreases clonogenic ability of MCF7 cells: (A) The cells were transfected with MITA and control vector or (B) co-transfected with p65shRNA or control random shRNA and clonogenic ability was analyzed as described in Materials and methods section. The colonies were stained with crystal violet for the assessment of clonogenic ability. (C) MITA was transfected in MCF-7 cells and migration ability of MCF-7 cells was checked by scratch assay as described in Materials and methods section. (D) MITA shRNA and control random shRNA were transfected in HBL100 cells and its migration ability was checked by scratch assay.

effective in the treatment of advanced soft tissue sarcoma [36]. Recently it has been shown that TNF-α expressing MDA-MB231 cells failed to form tumor *in vivo*. It also suggests that TNF-α interrupts symbiotic metabolic coupling between epithelial cancer cells and their host stromal microenvironment leading to death [37]. MITA down regulation in breast cancer tissue is a strategy of tumor to resist the antitumor effect of TNF-α.

The activation of cell death pathway strongly suggests that expression of MITA leads to decrease in the clonogenic ability. The migration ability of MCF-7 as well as HBL100 cells is also affected in the presence or absence of MITA respectively. The activation of caspase-8 is known to negatively regulate migration ability of the cells [38,39]. Caspase-8 binds to the lamella of the migrating cell and promotes the cell migration. Its catalytic activity is not required for the process. The decreased migration of MCF-7 cells upon MITA expression might be due to the increase in caspse-8 activity which ultimately makes pro-caspase-8 unavailable for binding and thus migration. Unraveling the mechanism of role of MITA in connecting these two observations is important to modulate the innate immune pathway for therapeutic intervention in breast cancer.

5. Conclusion

The current study provides strong evidences that MITA can act as potent tumor suppressor. MITA is significantly down regulated in breast cancer patients as well as in ER positive breast cancer cell line. The evidences in the current study suggest that MITA might prove to be an essential link to inflammation, endoplasmic reticulum and cancer. This

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hypothesis clearly warrants further study to understand link between inflammation and breast cancer.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.11.006.

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TLRs: Linking inflammation and breast cancer

Khyati Bhatelia, Kritarth Singh, Rajesh Singh *

Department of Bio-Chemistry, The M.S. University of Baroda, Vadodara 390002, Gujarat, India

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ABSTRACT

Breast cancer is one of the leading causes of mortality in the females. Intensive efforts have been made to understand the molecular mechanisms of pathogenesis of breast cancer. The physiological conditions that lead to tumorigenesis including breast cancer are not well understood. Toll like receptors (TLRs) are essential components of innate immune system that protect the host against bacterial and viral infection. The emerging evidences suggest that TLRs are activated through pathogen associated molecular patterns (PAMPs) as well as endogenous molecules, which lead to the activation of inflammatory pathways. This leads to increased levels of several pro-inflammatory cytokines and chemokines mounting inflammation. Several evidences support the view that chronic inflammation can lead to cancerous condition. Inflammation aids in tumor progression and metastasis. Association of inflammation with breast cancer is emerging. TLR mediated activation of NF-κB and IRF is an essential link connecting inflammation to cancer. The recent reports provide several evidences, which suggest the important role of TLRs in breast cancer pathogenesis and recurrence. The current review focuses on emerging studies suggesting the strong linkages of TLR mediated regulation of inflammation during breast cancer and its metastasis emphasizing the initiation of the systematic study.

Contents

1. Introduction

Breast cancer is the second most common cancer diagnosed worldwide. More than 1.3 million women worldwide are diagnosed with breast cancer each year [1]. Breast cancer rate has increased by 0.4% per year from 1975 to 1990; however its death rate decreased thereafter by 2.2% from 1990 to 2007 [2]. In spite of decrease in breast cancer incidence, about half-a-million women still die because of breast cancer each year [1,3]. The high figures of incidences and mortality, even with the advancement of primary screening and diagnosis, suggest the need to systematically investigate the cause and pathogenesis of breast cancer.

The physiological conditions that stimulate proliferation and growth of somatic cells leading to neoplasia and carcinoma are not well understood. The relationship between inflammation and cancer is emerging. The inflammatory diseases increase the risk of developing cancer [2,4–7]. For example, patients with ulcerative colitis and Crohn's disease are at increased risk for developing colorectal cancer [8]. Similarly,

* Corresponding author at: Department of Bio-Chemistry, Faculty of Science, The M.S. University of Baroda, Vadodara 390002, Gujarat, India. Tel.: +91 265 2759594.
E-mail address: singhraj1975@gmail.com (R. Singh).

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patients with schistosomiasis, stones, or long-term indwelling catheterization are prone to urinary bladder cancer and patients with atrophic gastritis are prone to gastric cancer [9]. The current review further describes the intricate relation between inflammation and cancer and further its role in the progression of the breast cancer.

The emerging evidences support the linkages between breast cancer and inflammation. Specific immune cells are permanent resident in the breast tissue such as macrophages [10,11]. Macrophages help in development and remodeling of the mammary gland [12–14]. The process of breast tumorigenesis is similar to tissue remodeling [15]. There is clear harmony between resident immune cells and mammary gland specific cells. Dysregulation of this equilibrium may lead to state of chronic inflammation; however, the causes and mechanisms are still not clear. The increased numbers of tumor associated macrophages (TAMs) are observed in the breast cancer tissue [10,11,16–18]. Macrophages, instead of protecting the host against diseased condition, facilitate the escape of tumor cells from resident immune system [19]. Pierce et al. reported that chronic inflammation plays a critical role in breast cancer recurrence [20,21]. Elevated levels of inflammatory markers such as C-reactive protein (CRP) and serum amyloid A (SAA) showed decreased cell survival in breast cancer patients irrespective of age, tumor stage, body mass index and race. The circulating CRP and SAA may be important prognostic markers in breast cancer patients. Inflammatory breast cancer (IBC) is one of the most aggressive types of breast cancer conditions, which strongly suggest that inflammation may be linked to breast cancer [22–25]. The chronic inflammatory condition due to infections also leads to the cancerous condition. *Helicobacter pylori* infection is associated with the development of gastric cancer [6]. These evidences suggest that chronic inflammatory conditions either due to the infections or alterations of physiological cancer are intricately linked to cancer. The molecular regulators of these linkages are emerging and will be discussed below.

1.1. TLRs: link between inflammation and cancer

TLRs are transmembrane receptors and essential part of host's innate immune system [26]. TLRs are generally present on immune cells; however, they are also expressed on epithelial cells, which come in the direct contact of pathogens. These are part of pattern recognition receptor family that recognizes PAMPs [27,28]. TLRs respond to two types of stimuli: exogenous and endogenous. PAMPs, exogenous stimuli of TLRs, are conserved molecular products derived from pathogens that include bacteria, fungi and viruses [29]. Danger associated molecular patterns (DAMPs) are endogenous molecules released from injured or dying cells; for example CpG nucleotide, dsDNA [30]. There are now established evidences that suggest that endogenous DAMPs bind to the TLRs in the manner similar to PAMPs and activate the downstream signaling pathways leading to activation of inflammatory pathways [31]. HSP60 was the first protein shown to act as ligand of TLR4 [32–34]; later, high mobility group protein (HMGB1) was observed to activate TLR2 and TLR4 [35]. The endogenous mRNA, ssRNA, and IgG/chromatin complexes are known to activate TLR3, TLR7, TLR8 and TLR9 respectively [31].

There is continuous oxidative stress in tumor microenvironment that leads to cellular damage and necrosis. This may also compromise the integrity of subcellular organelles like nucleus and mitochondria leading to the release of nuclear/mitochondrial DNA into the cytosol. The presence of mitochondrial DNA in the cytoplasm may bind to endosomal TLR9, activating the downstream pathway (Fig. 1). Mitochondrial DNA that escapes from autophagy is reported to activate TLR9 signaling pathway leading to chronic inflammation and subsequently causing heart failure [36]. The recent evidences also suggest that ER–mitochondria interface provides a novel platform to assemble novel inflammasome. NOD-like receptor protein 3 (NLRP3), ER–mitochondrial interface protein, senses DAMPs, which induce its oligomerization and subsequently activates caspase-1. This leads to maturation and secretion of IL-1β [37–39].

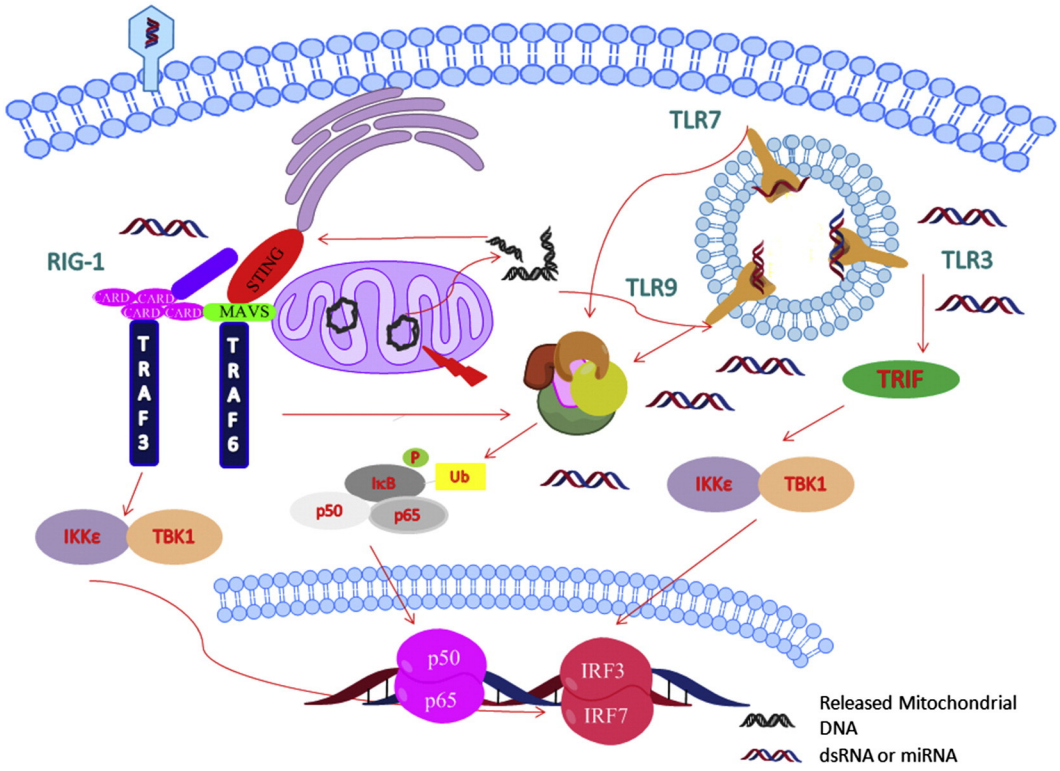

Fig. 1. Role of DAMPs in activation of inflammatory pathways: DAMPs such as dsDNA, dsRNA, and CpG nucleotides lead to the activation of inflammatory pathways by activating TLRs or other intracellular receptors like RIG-I and Nod like receptors. These signaling pathways lead to the activation of NF-κB and IFN mounting inflammation. The figure also represents possible endogenous molecules that may act as DAMP. During stress condition, mitochondrial DNA may be released in the cytosol which can also act as a potent DAMP.

Oxidized mitochondrial DNA released via programmed cell death activates NLRP3 inflammasome and releases IL1-β [40,41]. It has been also observed that stimulation of subset of TLRs (TLR1, TLR2 and TLR4) during infection also recruits the mitochondria at the site of intracellular pathogen and leads to generation of mitochondrial ROS (mROS) [41]. This suggests the linkage of TLR activation, mitochondrial ROS and IL-1β which raises an important question whether TLRs are activated in tumor microenvironment regulating IL-1β and mitochondrial ROS. Circulating mitochondrial DNA levels are observed to be significantly higher in the serum of cancer patients as compared to the control [42]. The increased levels of mitochondrial DNA may possibly act as ligand to TLR9, which affect the inflammatory pathways and promote tumorigenesis; however its relevance to the cancer including the breast cancer needs to be systematically investigated.

Pathogen or its molecular component interacts with toll like receptors (TLRs) on the surface of the cells and induces the signaling events that activate IRF or NF-κB transcription factor ultimately leading to expression of IRFs and NF-κB inducible genes that play critical role in innate immune response [31,43–47] (Fig. 2) [44,48,49]. This includes several inflammatory cytokines and chemokines expression [31]. These cytokines recruit and activate various leukocytes further amplifying the levels of proinflammatory cytokines [27,43,45]. They favor the process of angiogenesis and cell migration, which favor the tumor growth (Fig. 3). Pro-inflammatory cytokines like TNFα, IL-6 and IL-10 indeed induce tumor growth in specific conditions [50–55]. The increased levels of cytokine/chemokine either in inflammatory conditions or during infections lead to the expression of TLRs in cell types other than innate immune cells like tumor cells leading to their alterations in growth pattern and migration. TLRs are thus important mediators of chronic inflammatory conditions in tumor microenvironment providing survival advantage to the tumor cells.

1.2. TLR and breast cancer

The role of TLRs in breast cancer is not well understood; however emerging evidences clearly suggest strong linkages between them. The emerging role of TLRs in cancer progression has led breast cancer pathologist to systematically investigate the expression of TLRs in breast cancer tissue as well as cell lines [56]. The studies of TLRs in cell line and human tissue have clearly shown the important implication in the breast cancer.

1.3. TLR: the lessons from breast cell lines

The expression pattern of TLRs expressed is unique for each cell lines. MDA-MB-231, an epithelial breast cancer cell line derived from metastatic site pleural effusion, mimics the breast cancer cell properties in vitro. It is used as a model system to understand the process of metastasis of breast cancer at genetic level. It has been observed that MDA-MB-231 expressed all the TLRs at different levels, whereas TLR3 expression was the least [57]. The knockdown of TLR4 reduces cell viability in MDA-MB-231 [57]. The repression of TLR-4 mediated pro-inflammatory cytokines leads to reduced resistance of MDA-MB-231 against cytotoxic T lymphocyte and natural killer cell. Thus, inhibition or silencing of TLR4 in-vivo may be potential therapeutic modality to inhibit tumor growth [58,59]. The expression of TLR2 in MDA-MB-231 is 10.5 fold more than poorly invasive MCF-7 cells. The TLR2 activation leads to enhanced activity of NF-κB and increased levels of IL-6, TGF-β, VEGF and MMP9 [60]. This showed that the high invasiveness property is associated with TLR2 expression. Similarly, TLR9 activation by its agonist, CpG oligonucleotide, rendered MDA-MB-231 highly invasive, making it a good model to understand the metastasis and invasion of the breast cancer [61,62]. MCF7, an epithelial cell line is another model to study cancer

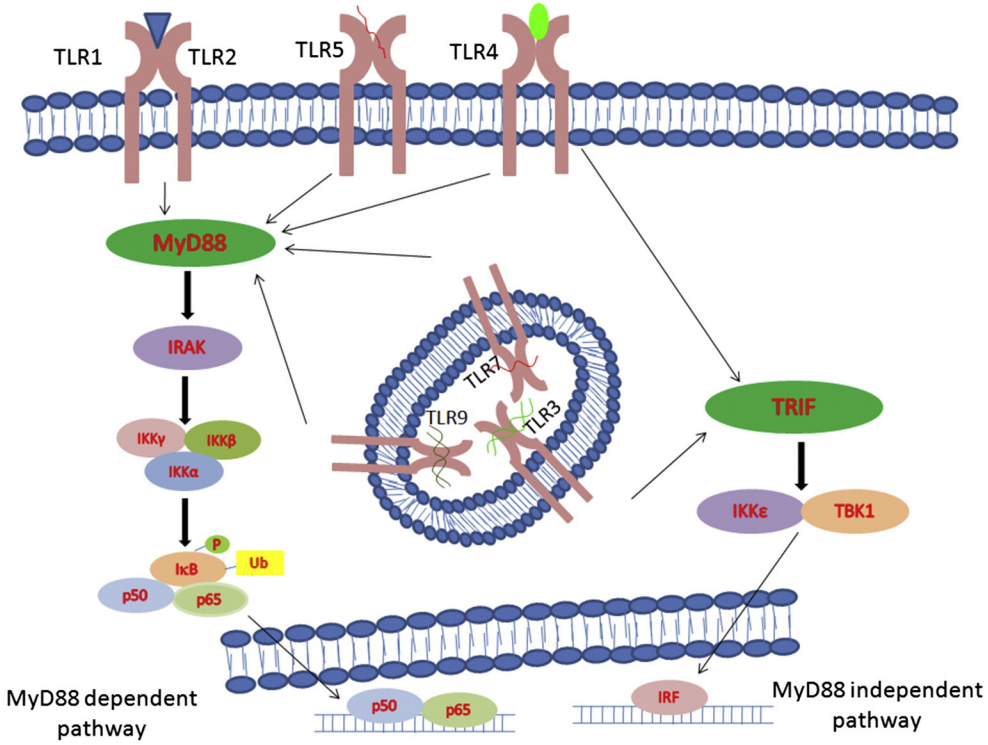

Fig. 2. TLR signaling pathway. TLR binds to its cognate ligand, it dimerizes and initiates downstream signaling pathways through one or more of four adaptor proteins: myeloid differentiation primary response gene 88 (*MyD88*), toll/interleukin-1-receptordomain-containing adaptor inducing interferon-β (*TRIF*), toll/interleukin-1-receptor-domain-containing adaptor protein (*TIRAP*), and TRIF-related adaptor molecule (*TRAM*). All TLRs, except for TLR3, initiate downstream signaling through MyD88. TLR3 signals through TRIF whereas TLR4 initiate signaling through both MyD88 and TRIF adaptor proteins. The binding of ligands to their cognate TLR leads to recruitment and auto-phosphorylation of serine/threonine kinases of the IL-1 receptor associated kinase (IRAK) family. This further activates downstream IKK complex, which lead to IκBα phosphorylation, ubiquitination and degradation. IκB is an inhibitor of NF-κB pathway. Its degradation leads to nuclear translocation of NF-κB and activates the transcription of the genes, encoding cytokines, chemokines and proteins involved in apoptosis regulation. In the case of MYD88 independent pathway, dsRNA bind to TLR3 receptor leading to TRIF recruitment. This further activates IKKε and TBK1 leading to phosphorylation of IRF3 and its nuclear translocation. This leads to activation of IFN inducible genes that play critical role in innate immune response.

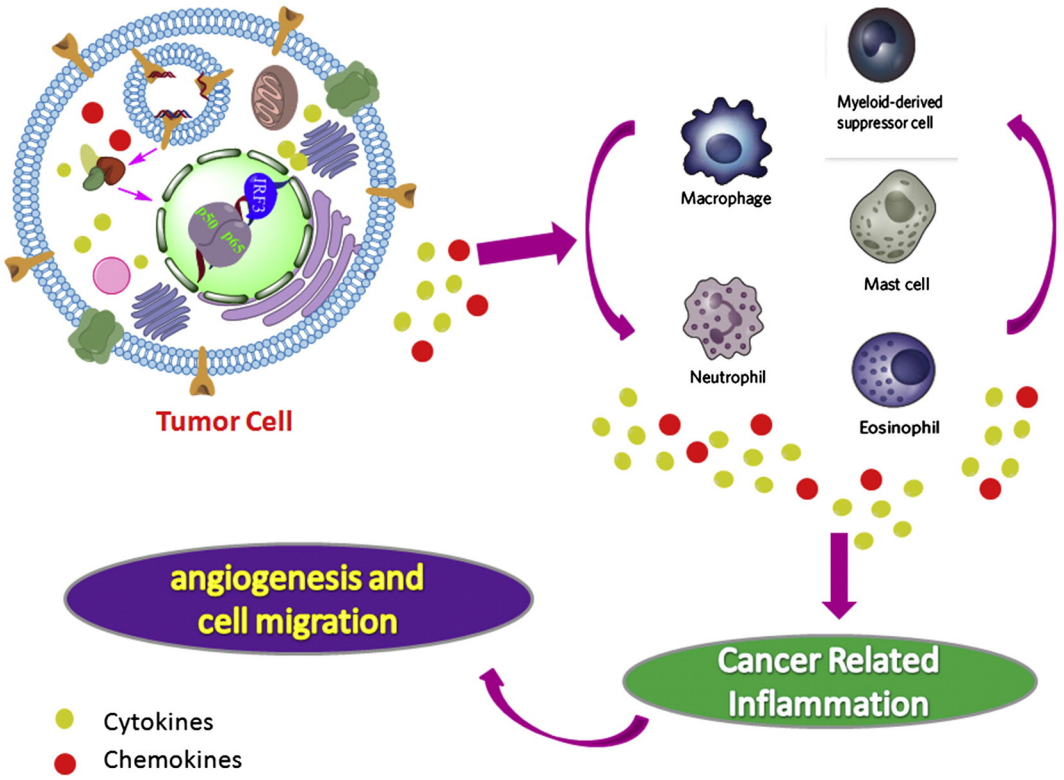

Fig. 3. Inflammation aids in tumor progression: Tumor cells have the ability to produce cytokine and chemokines by activating transcription factors such as NF-κB and IRFs. These cytokines and chemokines attract various immune cells to the tumor site. These cells in turn produce more cytokines and chemokines amplifying the whole process. This mounts cancer related inflammation and favors the process of angiogenesis and cell migration favoring tumor growth.

in vitro. The expression of different TLRs was observed at different levels in MCF-7 whereas TLR3 mRNA level was significantly higher [60]. The stimulation of TLR3 by its agonist dsRNA inhibits the cell growth and tumorigenic potential. It also sensitizes the cells to radiations by inducing autophagy mediated cell death [63]. The activation of TLR5 by bacterial flagellin inhibits cell proliferation in MCF7 cells as well as in mouse xenografts of human breast cancer cells by activating expression of CDK inhibitor p27 and decreasing cyclins and other soluble factors [64]. Similarly the activation of TLR3 by synthetic dsRNA induces apoptosis in Cama-1, human breast cancer cells which is TLR3/TRIF-dependent [65]. Similarly, TLR7 agonist, imiquimod, can elicit significant regression of spontaneous breast cancers in neu transgenic mice, a model of human HER-2/neu + breast cancer [66]. These evidences suggest that TLR mediated signaling plays an important role in regulation of cellular growth and death.

1.4. TLR implication in breast cancer metastasis: the patient studies

The studies in the breast cancer cell lines strongly suggest the role of TLRs in breast cancer; hence clinical studies to analyze the expression of TLRs and its implication in breast cancer were also initiated. TLR3, TLR4 and TLR9 have been reported to be highly expressed at mRNA level in patient samples of breast carcinomas. The patient tissues with recurrence show high expression of these TLRs relative to samples without recurrence. TLRs are not only expressed on tumor cells but they are also expressed on some stromal cells. TLR4 expression in mononuclear inflammatory as well as tumor cells was associated with higher metastasis. It was also observed that higher TLR9 expression on fibroblast like cells showed low metastasis [56,67]. In a study of more than 141 patients, TLR9 expression was observed to be positive in breast cancer epithelium tissue in more than 90% of breast cancer patients. The expression was predominantly in ER (−ve) tissue as compared to ER (+ve) tissue [68]. The relevance of TLR9 in breast cancer pathology is still not clear and needs to be investigated further. TLR3 is also reported to have a significant correlation with breast cancer pathogenesis. Amarante et al. analyzed the expression of TLR3, CXCR4 and IFNγ in the invasive breast cancer patients but observed no statistically significant difference; however mRNA levels of TLR3 were positively correlated with mRNA levels of CXCR4 and IFNγ. Most of the patients recruited under this study were in stage II and III of the breast cancer [69]. This suggests that TLR3 might be essential for invasiveness or metastasis of breast cancer.

TLR4 expression is also reported to be significantly correlated with the clinicopathological features of invasive ductal carcinoma, one of the most common types of breast cancer. In the study of nearly 50 patients, high TLR4 expression showed high incidence of lymph node metastasis [70]. Moreover, endotoxin/lipopolysaccharide, TLR4 ligand, promotes tumor cell adhesion and metastasis [71,72]. Triggering of TLR4 on metastatic breast cancer cells promotes integrin αvβ3 mediated adhesion and invasive migration of the cells by activating NF-κB [72]. $TLR4^{-/-}$ mice had increased tumor volume as well as higher rate of lung metastasis after injection of 4T1 tumor cells [73].

TLR polymorphisms specifically TLR2 and TLR4 are also associated with increased risk of cancer [74]. In a clinical report including 261 breast cancer patients and 480 healthy individuals, two of the polymorphism, Asp299Gly and Thr399Ile, of TLR4 and allelic frequencies of a 22-bp nucleotide deletion (−196 to −174 del) in the promoter of TLR2 gene were investigated [75]. Both of the TLR polymorphism showed increased susceptibility to the breast cancer [58,76]. The patients, having Asp299Gly polymorphism, with loss of function of TLR4, showed relapse earlier after anthracyclin based chemotherapy. TLR1 and TLR6 polymorphism, rs7696175, is also observed to be associated with increased risk of breast cancer in African American population [77,78].

TLR7 is considered to be an important tool for breast cancer therapeutic. Its agonist imiquimod efficiently inhibits tumor growth in mice. Its synergy with radiation therapy enhanced the inhibitory effect on tumor. Low dose of cyclophosphomide in conjunction with imiquimode and radiation therapy further improved tumor response [79]. These evidences suggest that TLRs have distinct expression and effect in different cell types. Interestingly, patient sample data and studies on breast cancer cell line contradict in case of TLR3 and TLR9; further study is needed to understand the TLR signaling mechanism in breast cancer cells. Reports also suggest that genetic alterations in TLR or NF-κB pathways are linked with increased risk of developing breast cancer [80]. Thus, most of the TLRs have profound effect on breast cancer and silencing or activating them might prove to be an effective therapeutic target for inhibiting the breast cancer.

The high rate of mortality in the breast cancer is associated with its progression to the distant metastatic site. Breast cancer tends to metastasize from its original site to the distant organs mainly lung, liver, bone and brain. Breast cancer metastasizing to brain has very less chances of survival, 20% probably for 1 year survival [81,82]. Breast to brain metastasis is also of interest because of the low survival rate and the limited success of chemotherapy due to the inability to cross the blood brain barrier [83]. Massague et al. have studied the genes involved in breast metastasis to lung, bone and brain. They have observed that MMPs, SOX4, and members in the TGFβ pathway are common in contributing to metastasis process [83–86]. The role of TLRs in metastasis to distant organ is not well studied, though evidences suggest their possible role in metastasis. The metastatic signature genes described above are regulated by TLRs in one or the other processes; for example TLR4 signaling augments TGF-β sensitivity that maintains and amplifies fibrosis in scleroderma [87]. TLRs also regulate MMPs, chemokines and cytokines, some of which are observed in contributing to breast cancer metastasis. Moreover, angiogenesis is a crucial process in dissemination and spreading of the metastatic cells. VEGF (vascular endothelial growth factor) plays an important role in angiogenesis, which is regulated by TLR2 [88]. Thus studying the role of TLRs in metastasis is of immense importance.

1.5. TLR and NF-κB: linking inflammation and breast cancer

The regulation of NF-κB helps in explaining the linkages of inflammation and cancer at molecular level [89]. NF-κB dysregulation has been observed in many of the cancers [89,90]. Inhibition of NF-κB leads to the apoptosis of transformed hepatocytes [91]. NF-κB transcription factor stimulates several genes, which play an important role in cell survival, resistance to the cell death, migration and angiogenesis (Fig. 4). The mechanisms of NF-kB activation that lead to unique outcome, either cellular proliferation or inhibition of apoptosis, and oncogenesis are not well understood [92]. Reports suggest that different types of TLRs are over-expressed in different cancers. High levels of TLR4 and TLR9 detected on lung cancer cells are functionally associated with higher tumor invasiveness and metast of asizing potential as well as anti-apoptotic activity [93,94]. TLR4 identified on ovarian cancer cells promote chemoresistance to paclitaxel [95,96]. The specific role of TLR mediated cellular process like growth, migration and resistance/sensitivity to death is emerging in different cancers including the breast cancer.

The alteration in the expression of TLRs during cancer, including breast cancer, is observed frequently. The alterations of TLRs expression lead to dysregulation of downstream mediators of TLR pathway. NF-κB is one of such downstream regulator of TLRs as well as other intracellular receptors important in inflammation. As described above, NF-κB regulates the expression levels of pro/anti inflammatory cytokine that play a key role in survival of the tumor cell. ER resident protein MITA (mediator of IRF3 activation) regulates NF-κB during viral infection. We recently reported that MITA acts as tumor suppressor in breast cancer. It is down regulated in breast tumor tissues. MITA expression in breast cancer cell line activates NF-κB, which in turn induces TNF. MITA also sensitizes TNF induced cell death in breast cancer cell lines, by activating caspases (Fig. 5) [97].

The association of NF-κB and breast cancer is further emphasized by the observation of amplification and over-expression of IKKε, a central kinase in NF-κB pathway, in breast cancer cell lines and patient-

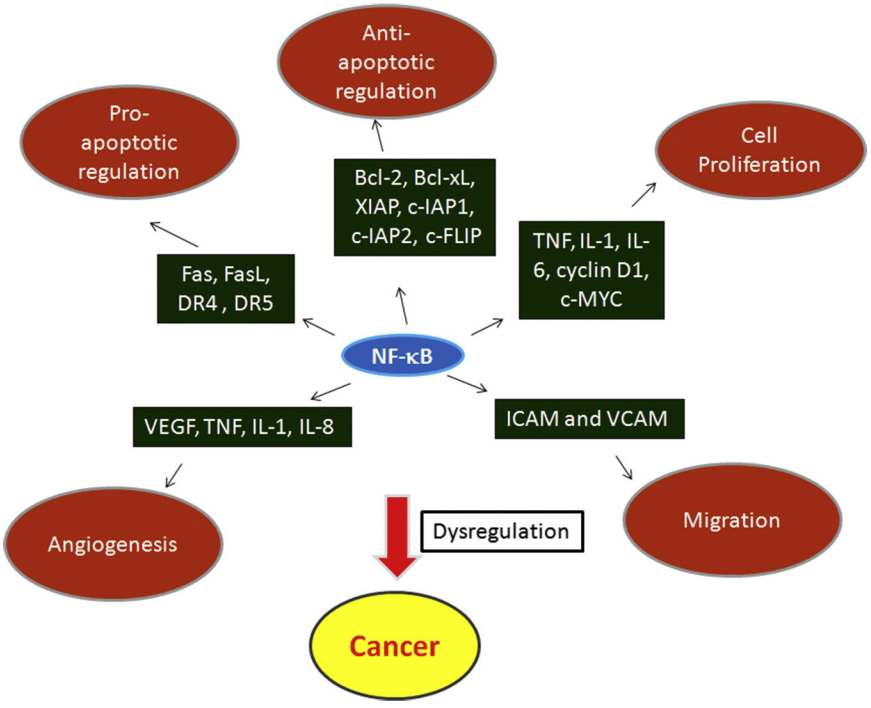

Fig. 4. NF-κB regulator of cancer related pathways. NF-κB regulates the expression of anti-apoptotic genes such as Bcl-2, Bcl-xL, XIAP, c-IAP1, c-IAP2, c-FLIP. At the same time it regulates the expression of pro-apoptotic genes such as Fas, FasL, DR4 (Death Receptor 4), DR5. It also regulates the expression of adhesion molecules such as ICAM and VCAM, dysregulation in which aids in tumor migration. NF-κB regulated TNF, IL-1, IL-6, cyclin D1, c-MYC and others lead to cell proliferation and VEGF, TNF, IL-1, and IL-8 aid in angiogenesis that favors the process of tumorigenesis.

derived tumors [98]. Over expression of IKKε is observed in 30% of the breast cancers cell lines and carcinomas [98,99]. The significance and molecular basis of IKKε overexpression in breast cancer are however under investigation. DNA damage induces kinase dependent IKKε translocation to the nucleus where it co-localizes with the TOPORs and gets summoylated. This sumoylated form of IKKε phosphorylates and activates NF-κB transcription factor leading to cell death or survival [100]. During DNA damage, release of nucleotides or DNA complexes in the cytosol cannot be ruled out which may activate the endogenous TLRs such as TLR7 and TLR9 as well as cytosolic sensors such as MITA which may possibly activate IKKε [101]. It will be interesting to study the modulation of TLRs in IKKε over expressing breast tumor. IKKε, a downstream mediator of RIG-1 pathway, is also known to play an important role in antiviral response [102]. As discussed above, DAMPs, like miRNA or endogenous dsRNA, may also activate TLR3 or RIG1 pathway to positively regulate IFN pathway. Recent report demonstrates that the component pathway of TLR3/RIG pathway is downregulated in liver tumors [103]. The potential role of the activation of endosomal TLR3 and RIG1 pathway in cancer specifically breast cancer is not well studied.

1.6. TLR: implication in therapeutics of breast cancer

The best strategy to manage later stage of breast cancer is surgery, which is generally followed by systematic therapies such as chemotherapy, radiation, hormone or targeted therapies. These therapies have met with limited success. There is need for extensive understanding of the pathogenesis and its associated altered physiological conditions, to design better therapeutic strategies to manage cancer.

Hormonal therapy for breast cancer includes competitive inhibition of the hormone receptor binding by its analog molecule. The estrogen receptor positive and progesterone receptor positive breast cancer subtypes respond to this therapy; whereas others are not responsive to hormonal therapies. Chemotherapy induces the cell death in tumor cells, but it fails to distinguish between cancerous and normal cells; hence, the collateral non specific cell death is observed. This strongly suggests the development of targeted therapies to target only cancer cells. Tratuzumab or herceptin are monoclonal antibodies that bind to Her2 receptor and targets Her2 amplified tumor cells [24,104–107]. Her2 overexpression enhances cell survival by activating NF-κB. Hence Her2 monoclonal antibodies are used in combination with chemotherapy or radiotherapy. The use of herceptin sensitizes Her2 positive cells to chemotherapy and radiotherapy by modulating NF-κB pathway.

Triple negative breast cancer subtype (also known as ER/PR/Her2 negative tumor) does not express ER/PR/Her2 receptors. Triple negative subtype lacks tumor specific marker making it hard to deliver a drug in targeted manner. The only treatment available for this subtype of tumor is chemotherapy and surgery; however the success rate is 77% as compared to 93% of other subtypes [1]. Recently, one of the reports showed that concurrent inhibition of IL-6 and IL-8 inhibited colony forming ability and cell survival in vitro and restricted tumor growth in vivo [108]. Involvement of inflammatory cytokines in facilitating tumor growth of TNBC subtype suggests that TLR mediated therapies might prove to be an effective cure to breast cancer of triple negative subtype.

Several TLR agonists are being tried as therapeutics for cancer [109–111]. Polysaccharide krestin (PSK), a mushroom extract that has been long used as a treatment for cancer, has been shown to be a TLR2 agonist. PSK significantly inhibits tumor growth in neu transgenic mice; but PSK fails to inhibit tumor growth in TLR2 knockout mice [112,113]. The effect of PSK is dependent on the CD8 + T cells and NK cells which in turn depend on TLR2. The other possible TLR ligand having antitumorigenic potential is TLR-7 agonist, imiquimod. Topical treatment with imiquimod leads to significant regression of spontaneous breast cancers in neu transgenic mice, a model of human HER-2/neu + breast cancer [66]. The effect of TLR7 agonist ended once the agonist treatment was stopped as it increased IL-10 expression in addition to IFNβ and TNFα. Blockade of IL-10 enhanced imiquimode induced antitumor activity in mice. TLR9 agonist, CpG oligodeoxynucleotides (ODN), is also emerging as cancer therapeutics alone or in combination with chemotherapy or radiation therapy, though its clinical relevance with breast cancer needs to be studied [114]. Studying the relation between TLR and breast cancer is thus of interest for diagnosis as well as prognosis of the breast cancer.

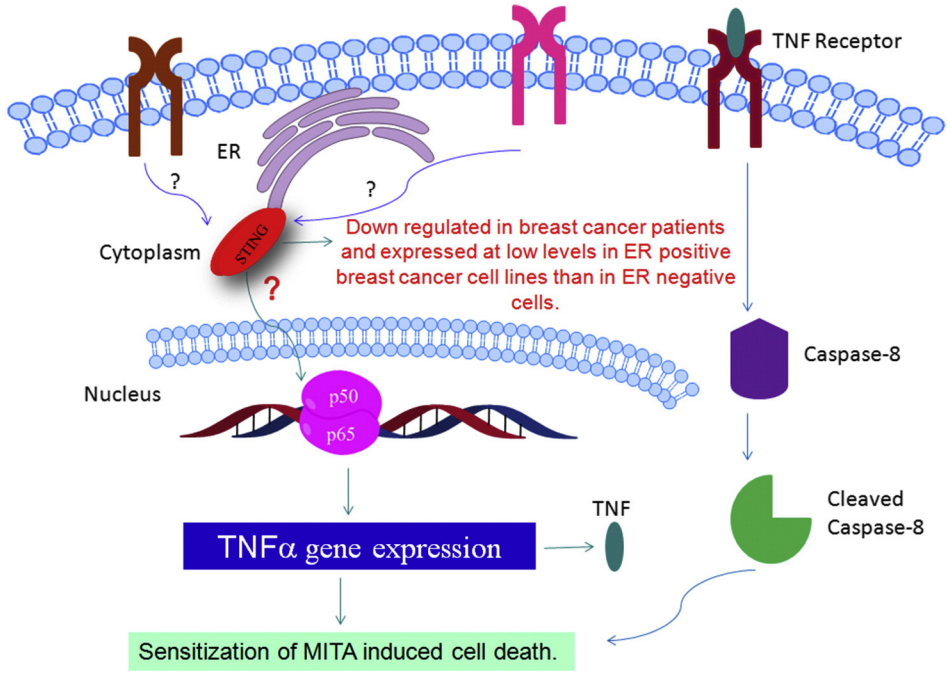

Fig. 5. MITA acts as a tumor suppressor in breast cancer. MITA is down-regulated in breast cancer patient as well as cell lines. MITA has potential to induce cell death by activating NF-κB through an unknown mechanism in breast cancer cells. NF-κB activation leads to the expression of TNF-α. MITA induced cell death is sensitized by TNF treatment which involves the active role of caspase-8.

1.7. Type-I IFNs: emerging therapeutic for breast cancer

Type-I IFNs have been indeed used for the treatment of hematological malignancies or some solid tumors, for example, melanoma, renal carcinoma, and Kaposi's sarcoma [115]. It was first discovered as an antiviral agent about 50 years ago; later it was observed to inhibit the growth of tumor cells and inhibit cellular transformation by viruses [116]. The observation excited the scientists to employ IFNs as anticancer treatment and clinical trials were initiated. IFNα is in phase III clinical trial against advanced renal cell carcinoma [117,118]. IFN gene therapy is also being studied in breast cancer. Human breast cancer xenografts have been treated by expressing IFN using adenovirus vector [119]. Recently IFN-β is used in fusion with antioncogenic receptor antibodies to overcome the limitation of Ab resistance seen in various tumors [120]. IFN therapy still has side effects, which need to be considered before proposing IFN as potential therapeutics. IFNs play an important role in the immune system and regulation of inflammation. IFNs when given to patients lead to autoimmune response unnecessary inflammatory reaction [115]. It also leads to tissue toxicity and hence needs to be replaced by targeted therapy. The possibility to stimulate the cancer cells themselves to produce IFNs can be investigated as another alternative for targeted therapies. TLRs play a critical role in regulation of IFN and NFκB; hence stimulating these pathways might prove to be an effective IFN treatment. It needs extensive studies to understand the molecular mechanisms involved in these pathways as well as how cancer cells have evolved these mechanisms for their benefits.

Other than IFNs, other cytokines regulated by TLR pathways are also being employed in breast cancer treatment. IL-2, IFNα, IFNβ, IL-6 and IL-12 have been tried for the treatment of advanced stages of breast cancer [121,122]. Receptor of IL-8, CXCR1, is highly expressed on breast cancer stem cells. Blocking of this receptor in mouse xenograft reduces the numbers of these stem cells [123]. These observations suggest the possible use of TLR agonist/antagonist to modulate the breast tumorigenesis.

2. Conclusions

The emerging evidences clearly suggest that inflammation and breast cancer are intricately linked. TLRs are the key players in activating inflammatory pathways and creating favorable tumor microenvironment by producing various cytokines and chemokines promoting the tumor growth. It will be interesting to understand the multiple TLR expression on the typical breast tumor cell type and their probable role in tumorigenesis.TLR pathway involves numerous proteins, which are known potential oncogenes/tumor suppressor like IKKε and MITA. Studying the role of such other proteins of TLR pathway in breast tumorigenesis will further help to understand the significance of TLR pathway in tumorigenesis including breast cancer. The metastasis of breast cancer to the different organs during relapse is an emerging problem in the aging breast cancer population. The role of TLR regulatory process in the regulation of metastasis is emerging. The study of these mechanisms will help to modulate TLRs for therapeutic purpose. Modulation of these TLRs in tumor cells might help secreting specific cytokines having antitumoric effect such as IFNs and TNFα. These cytokines might act in autocrine or paracrine manner to stimulate nearby tumor cells, thus inhibiting the tumor growth. It would be interesting to study the mechanism of miRNA mediated regulation of these TLRs or other proteins of TLR pathway. There are several questions which need to be answered for understanding the implications of TLRs and their therapeutic modulation in breast cancer.

Conflict of interest

The authors declare that there is no conflict of interest.

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NLRX1 acts as tumor suppressor by regulating TNF-α induced apoptosis and metabolism in cancer cells

Kritarth Singh [a,b], Anastasia Poteryakhina [c], Andrei Zheltukhin [c], Khyati Bhatelia [a,b], Paresh Prajapati [a,b], Lakshmi Sripada [a,b], Dhanendra Tomar [a], Rochika Singh [a], Arun K. Singh [a], Peter M. Chumakov [c,*], Rajesh Singh [a,**]

[a] Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India
[b] Department of Cell Biology, School of Biological Sciences and Biotechnology, Indian Institute of Advanced Research, Gandhinagar, India
[c] Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia

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ABSTRACT

Chronic inflammation in tumor microenvironment plays an important role at different stages of tumor development. The specific mechanisms of the association and its role in providing a survival advantage to the tumor cells are not well understood. Mitochondria are emerging as a central platform for the assembly of signaling complexes regulating inflammatory pathways, including the activation of type-I IFN and NF-κB. These complexes in turn may affect metabolic functions of mitochondria and promote tumorigenesis. NLRX1, a mitochondrial NOD-like receptor protein, regulate inflammatory pathways, however its role in regulation of cross talk of cell death and metabolism and its implication in tumorigenesis is not well understood. Here we demonstrate that NLRX1 sensitizes cells to TNF-α induced cell death by activating Caspase-8. In the presence of TNF-α, NLRX1 and active subunits of Caspase-8 are preferentially localized to mitochondria and regulate the mitochondrial ROS generation. NLRX1 regulates mitochondrial Complex I and Complex III activities to maintain ATP levels in the presence of TNF-α. The expression of NLRX1 compromises clonogenicity, anchorage-independent growth, migration of cancer cells in vitro and suppresses tumorigenicity in vivo in nude mice. We conclude that NLRX1 acts as a potential tumor suppressor by regulating the TNF-α induced cell death and metabolism.

1. Introduction

Clinical and experimental studies suggest that inflammation is intricately linked with tumorigenesis. In colorectal, hepatic, breast and several other cancer types, an inflammatory condition may precede the development of malignancy [1–3]. For example, inflammatory bowel disease (IBD) is associated with colon cancer and an infection by *Helicobacter pylori* progressively leads to gastric carcinoma [3,4]. However, despite the numerous examples of the apparent association of chronic inflammatory conditions with higher incidences of cancer, the molecular mechanisms linking these pathologies are still not well understood.

Inflammation, irrespective of its origin, promotes cell survival, proliferation of malignant cells and conditions the tumor microenvironment for further metastasis. Emerging clinical reports suggest that the levels of specific cytokines are altered in patients with different cancer types including breast, gastric, colorectal and hepatocellular carcinomas [5]. Increased levels of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), macrophage migration inhibitory factor (MIF), transforming growth factor beta (TGF-β), interleukins-6, -8, -10 and -18 (IL-6, IL-8, IL-10 and IL-18) were reported in patients with advanced-stage pancreatic, colorectal and breast cancers [6–15]. Serum levels of TNF-α were elevated in eight independent types of cancer including breast, colorectal and gastric carcinomas [5,9,13]. In tumor microenvironment, TNF-α secreted by tumor cells or by inflammatory cells, promotes tumor cell survival through the stimulation of NF-κB pathway [16]. The activation of NF-κB up-regulates the expression of genes stimulating cell cycle progression and promotes epithelial–mesenchymal transition [17]. The binding of TNF-α to Type I TNF receptor (TNFR1) results in a pro-survival stimulation of NF-κB, through the formation of proximal plasma membrane bound complex I consisting of TNF receptor-associated protein with death domain (TRADD), receptor-interacting protein 1 (RIP1) and TNF receptor-associated factor 2 (TRAF2). During the TNF-α induced apoptosis, the complex-I dissociates from TNFR1 and recruits the Fas-associated death domain (FADD) and Caspase-8, forming cytosolic complex-II, where Caspase-8 is activated, which further initiates the downstream proteolytic cascade

* Correspondence to: P.M. Chumakov, Cell Proliferation Laboratory, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilov Street 32, Moscow, Russia. Tel.: +7 499 135 2331; fax: +7 499 135 1405.
** Correspondence to: R. Singh, Department of Biochemistry, Faculty of Science, The M.S. University of Baroda, Vadodara, Gujarat, India. Tel.: +91 265 2795594.
E-mail addresses: peter@chumakov.com (P.M. Chumakov), singhraj1975@gmail.com (R. Singh).

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TNF-α regulates miRNA targeting mitochondrial complex-I and induces cell death in dopaminergic cells

Paresh Prajapati [a,b], Lakshmi Sripada [a,b], Kritarth Singh [a,b], Khyati Bhatelia [a,b], Rochika Singh [a], Rajesh Singh [b,*]

[a] Department of Cell Biology, School of Biological Sciences and Biotechnology, Indian Institute of Advanced Research (IIAR), Koba Institutional Area, Gandhinagar 382007, Gujarat, India
[b] Department of Biochemistry, Faculty of Science, The M. S. University of Baroda, Vadodara 390002, Gujarat, India

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ABSTRACT

Parkinson's disease (PD) is a complex neurological disorder of the elderly population and majorly shows the selective loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc) region of the brain. The mechanisms leading to increased cell death of DAergic neurons are not well understood. Tumor necrosis factor-alpha (TNF-α), a pro-inflammatory cytokine is elevated in blood, CSF and striatum region of the brain in PD patients. The increased level of TNF-α and its role in pathogenesis of PD are not well understood. In the current study, we investigated the role of TNF-α in the regulation of cell death and miRNA mediated mitochondrial functions using, DAergic cell line, SH-SY5Y (model of dopaminergic neuron degeneration akin to PD). The cells treated with low dose of TNF-α for prolonged period induce cell death which was rescued in the presence of zVAD.fmk, a caspase inhibitor and N-acetyl-cysteine (NAC), an antioxidant. TNF-α alters mitochondrial complex-I activity, decreases adenosine triphosphate (ATP) levels, increases reactive oxygen species levels and mitochondrial turnover through autophagy. TNF-α differentially regulates miRNA expression involved in pathogenesis of PD. Bioinformatics analysis revealed that the putative targets of altered miRNA included both pro/anti apoptotic genes and subunits of mitochondrial complex. The cells treated with TNF-α showed decreased level of nuclear encoded transcript of mitochondrial complexes, the target of miRNA. To our knowledge, the evidences in the current study demonstrated that TNF-α is a potential regulator of miRNAs which may regulate mitochondrial functions and neuronal cell death, having important implication in pathogenesis of PD.

1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative disorder, affecting millions of elderly individuals worldwide [1,2]. The increase in aging population is already showing exponential rise in PD cases. The mechanisms leading to PD had been the focus of research for the last several years; however, there is no effective therapy or any potential marker for monitoring the progression of PD. Neuropathological examination of the post-mortem brain suggests that several regions of the brain are affected, however the loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc) is one of the most prominent features of PD [3]. At the time of clinical presentation approximately 50–70% of DAergic neurons in the nigrostriatal system are already lost [4]. The mechanisms leading to degeneration of DAergic neurons are still not well understood.

Inflammation and its association with neurodegenerative diseases are emerging [5,6]. Several studies provide strong evidences for the association of inflammation with sporadic and familial forms of the PD. The studies of post-mortem human brain obtained from PD patients provided direct evidence of the association with inflammation with PD. HLA-DR-positive reactive microglia were clearly observed within the substantia nigra of PD patients [7]. The increased levels of several pro-inflammatory cytokines (IL1-β, IL-2, IL-6, TNF-α and IFN-γ) were observed in the DAergic nigrostriatal system and the regions outside the SN in PD patients [8–13]. TNF-α is one of the important pleiotropic cytokines and had been implicated in both neuronal survival and death. TNF-α is known to induce ROS (reactive oxygen species) generation in mitochondria [14]. The mitochondrial complex I and complex III are the primary sites of ROS generation. The homeostasis of mitochondria is maintained through selective elimination of defective mitochondria by the process of selective autophagy called as mitophagy [15]. The role of TNF-α in regulation of mitochondrial dysfunction, generation of ROS and implication in mitophagy during PD conditions is not well understood.

The optimal functioning of mitochondria requires more than 1000 proteins. Hence >1000 resident proteins and critical non-coding RNAs (RNaseP, RNA component of MRP and 5S rRNA) are encoded from nuclear genome and are imported into mitochondria for their optimal function [16]. The miRNAs, emerging class of small non-coding RNAs, play important role in the regulation of mRNA copy number and protein level in the narrow physiological range [17]. Recently, our group

* Corresponding author. Tel.: +91 265 2795594.
E-mail address: singhraj1975@gmail.com (R. Singh).

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ORIGINAL ARTICLE

hsa-miR-4485 regulates mitochondrial functions and inhibits the tumorigenicity of breast cancer cells

Lakshmi Sripada[1] · Kritarth Singh[1] · Anastasiya V. Lipatova[2] · Aru Singh[3] · Paresh Prajapati[1] · Dhanendra Tomar[4] · Khyati Bhatelia[1] · Milton Roy[1] · Rochika Singh[5] · Madan M. Godbole[3] · Peter M. Chumakov[2] · Rajesh Singh[1]

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Abstract The modulation of mitochondrial functions is important for maintaining cellular homeostasis. Mitochondria essentially depend on the import of RNAs and proteins encoded by the nuclear genome. MicroRNAs encoded in the nucleus can translocate to mitochondria and target the genome, affecting mitochondrial function. Here, we analyzed the role of miR-4485 in the regulation of mitochondrial functions. We showed that miR-4485 translocated to mitochondria where its levels varied in response to different stress conditions. A direct binding of miR-4485 to mitochondrial 16S rRNA was demonstrated. MiR-4485 regulated the processing of pre-rRNA at the 16S rRNA-ND1 junction and the translation of downstream transcripts. MiR-4485 modulated mitochondrial complex I activity, the production of ATP, ROS levels, caspase-3/7 activation, and apoptosis. Transfection of a miR-4485 mimic downregulated the expression of regulatory glycolytic pathway genes and reduced the clonogenic ability of breast cancer cells. Ectopic expression of miR-4485 in MDA-MB-231 breast carcinoma cells decreased the tumorigenicity in a nude mouse xenograft model. Furthermore, levels of both precursor and mature miR-4485 are decreased in tumor tissue of breast cancer patients. We conclude that the mitochondria-targeted miR-4485 may act as a tumor suppressor in breast carcinoma cells by negatively regulating mitochondrial RNA processing and mitochondrial functions.

Keywords Mitochondria · miR-4485 · Breast cancer · Tumor suppressors · RNA processing · Mouse xenograft

Introduction

Mitochondria are indispensable for energy production, lipid and carbohydrate metabolism, redox regulation, calcium signaling, and cell death. Mitochondria have also been implicated in the regulation of innate immunity, inflammation, and antiviral signaling [1, 2]. Mitochondrial dysfunction is associated with numerous pathologies, including metabolic and neurodegenerative disorders, cardiomyopathies, cancer, and aging [3, 4]. Reprogramming of mitochondrial functions is one of the major hallmarks of tumor cell metabolism [5, 6]. To cope with growing bioenergetic demands of rapid proliferation, cancer cells can switch from an efficient but slow mitochondrial respiration to the less efficient but rapid aerobic glycolysis [7–10]. Some of the key intermediates, such as citrate and glycerol, are redirected from the Krebs cycle to meet increased demands of tumor cells in macromolecular synthesis [11, 12]. Although mechanisms of metabolic reprogramming in rapidly dividing cancer cells are being extensively studied, many of the processes remain elusive.

Proteomics studies have revealed that the human mitochondrion contains more than a thousand distinct

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✉ Rajesh Singh
singhraj1975@gmail.com

[1] Department of Biochemistry, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat 390002, India

[2] Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia

[3] Department of Endocrinology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, Uttar Pradesh 226014, India

[4] Center for Translational Medicine, Temple University, Philadelphia, PA 19140, USA

[5] Department of Cell Biology, School of Biological Sciences and Biotechnology, Indian Institute of Advanced Research, Koba Institutional Area, Gandhinagar, Gujarat 382007, India

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Original contribution

TRIM4; a novel mitochondrial interacting RING E3 ligase, sensitizes the cells to hydrogen peroxide (H_2O_2) induced cell death

Dhanendra Tomar [a,b,1], Paresh Prajapati [c], Julie Lavie [b], Kritarth Singh [c], Sripada Lakshmi [c], Khyati Bhatelia [c], Milton Roy [c], Rochika Singh [a], Giovanni Bénard [b,*], Rajesh Singh [c,*]

[a] Department of Cell Biology, School of Biological Sciences and Biotechnology, Indian Institute of Advanced Research, Gandhinagar, India
[b] Université de Bordeaux, Laboratoire Maladie Rares: Genetique et metabolisme, Hopital Pellegrin, 33076 Bordeaux, France
[c] Department of Biochemistry, Faculty of Science, The M.S. University of Baroda, Vadodara 390002, Gujarat, India

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ABSTRACT

The emerging evidences suggest that posttranslational modification of target protein by ubiquitin (Ub) not only regulate its turnover through ubiquitin proteasome system (UPS) but is a critical regulator of various signaling pathways. During ubiquitination, E3 ligase recognizes the target protein and determines the topology of ubiquitin chains. In current study, we studied the role of TRIM4, a member of the TRIM/RBCC protein family of RING E3 ligase, in regulation of hydrogen peroxide (H_2O_2) induced cell death. TRIM4 is expressed differentially in human tissues and expressed in most of the analyzed human cancer cell lines. The subcellular localization studies showed that TRIM4 forms distinct cytoplasmic speckle like structures which transiently interacts with mitochondria. The expression of TRIM4 induces mitochondrial aggregation and increased level of mitochondrial ROS in the presence of H_2O_2. It sensitizes the cells to H_2O_2 induced death whereas knockdown reversed the effect. TRIM4 potentiates the loss of mitochondrial transmembrane potential and cytochrome c release in the presence of H_2O_2. The analysis of TRIM4 interacting proteins showed its interaction with peroxiredoxin 1 (PRX1), including other proteins involved in regulation of mitochondrial and redox homeostasis. TRIM4 interaction with PRX1 is critical for the regulation of H_2O_2 induced cell death. Collectively, the evidences in the current study suggest the role of TRIM4 in regulation of oxidative stress induced cell death.

1. Introduction

The studies in the last two decades suggest that beside metabolism, mitochondria plays crucial role in other cellular processes like cell death, inflammation and differentiation [1–3]. The regulation of mitochondrial function is required for cellular homeostasis and its dysregulation had been implicated in various pathological conditions like neurodegeneration, ageing, inflammation, infection and cancer [4–6]. The understanding of the regulation of mitochondrial functions is important to modulate its function in associated pathological condition.

Mitochondria are one of the primary sites of the production of reactive oxygen species (ROS) during physiological and pathological conditions [7,8]. The regulated level of ROS plays critical role in different cellular processes like cell cycle, proliferation, differentiation, migration [9–11]; however, its excess leads to the activation of cell death pathways [12,13]. The physiological level of ROS is maintained by redox reactions and activity of several antioxidant enzymes like glutathione peroxidases (GPX), thioredoxins (TRX) and peroxiredoxins (PRX) [14–16]. PRXs are member of low molecular weight peroxidases, involved in regulation of redox signaling [16]. PRX scavenge low concentrations of H_2O_2, hence acts as modulator of H_2O_2 signaling [16,17]. The regulation of different antioxidant enzymes and their selective role in oxidative stress induced cell death is less understood.

The emerging evidences suggest that ubiquitin mediated post-translational modifications plays critical role in the regulation of redox pathways [18,19]. The ubiquitin E3 ligases are terminal protein during ubiquitination and provide specificity to this process as it recognizes the substrate and transfer Ub moiety to the target [20]. Ubiquitin E3 ligase, E6AP, regulates the cellular response during oxidative stress condition by modulating the turnover of PRX1 [21]. The role of specific E3 ligase, their recruitment to mitochondria and regulation of redox signaling, cell death during oxidative stress is less understood.

TRIM proteins are members of RING family of ubiquitin E3

* Corresponding authors.
E-mail addresses: giovanni.benard@inserm.fr (G. Bénard), singhraj1975@gmail.com (R. Singh).
[1] Present Address: Center for Translational Medicine, Temple University, Philadelphia, PA 19140, USA.

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