7. Mitochondrial DNA aligned miRNA: miR-4485

The association, import and role of novel miRNAs into mitochondria have been demonstrated. Interestingly, miR-4485 has sequence homology at 16S rRNA region in the mitochondrial genome. In this chapter, the association and transport of hsa-miR-4485 into mitochondria and its role in mitochondrial function, cell death and tumorigenicity has been elucidated.

7.1 Alignment of small RNA to human mitochondrial genome

Human mitochondria is known to contain 16.5 kb circular DNA as its own genome. We determined sRNA alignment to the mitochondrial genome (Sano et al., 2008) to identify sRNA that may regulate mitochondrial gene expression or may be encoded by mitochondrial genome.

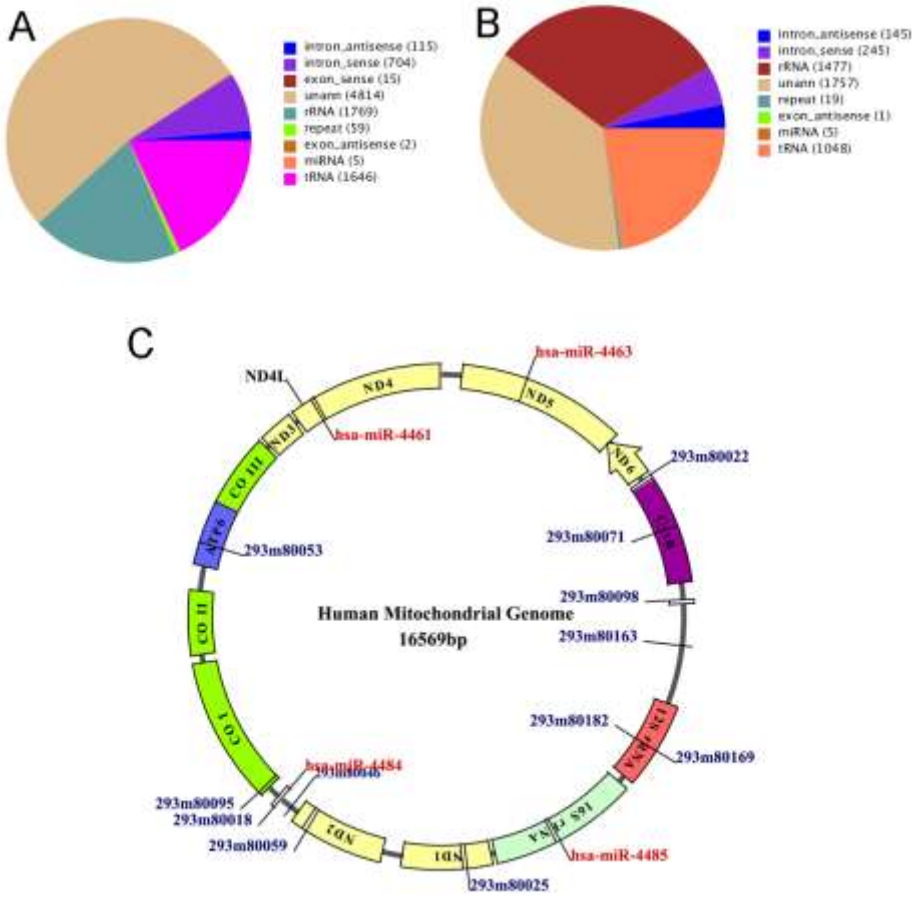

Figure 7.1. Mapping sRNAs to human mitochondrial genome. *The sequences obtained from the mitochondrial sRNA libraries from HEK293 and HeLa were aligned to mitochondrial genome. (A) An overview of mitochondria-associated sRNAs from HEK293 that aligned to mitochondrial genome. (B) An overview of mitochondria-associated sRNAs from HeLa that aligned to mitochondrial genome. (C) The miRNAs and putative novel miRNAs that aligned to mtDNA (determined by SOAP, MapMi, BLASTN and RNAhybrid) were mapped on mtDNA using Dynamo Software tool. The locations of known miRNAs and putative novel miRNAs that aligned to mtDNA are marked in red and blue respectively. If more than 1 miRNA aligned to same position, only 1 miRNA was marked.*

We used two different approaches to search for the sequences that aligned to mitochondrial genome. Using SOAP parameters described in method section, 4697 unique sequences (corresponding to 14102 reads) from HEK293 and 9129 unique sequences (corresponding to 78374 reads) from HeLa libraries aligned to human mitochondrial genome. The class of the above matched sequences belonged to unannotated (40.9% in HEK293 and 16.8% in HeLa), tRNA (38.2 % in HEK293 and 40.1% in HeLa), rRNA (16.4% in HEK293 and 39.4% in HeLa) categories. Few sequences were also categorized to be repeat associated sequences (0.36% in HEK293 and 0.14% in HeLa) and miRNA (0.01% in HEK293 and 0.035% in HeLa) (Figure 7.1A, Figure 7.1B). The analysis showed that only four miRNA: hsa-miR-4461, hsa-miR-4463, hsa-miR-4484 and hsa-miR-4485 aligned to mitochondrial genome at positions (10690 - 10712), (13050 - 13068), (5749 - 5766) and (2562 - 2582) on ND4L, ND5, L-ORF and 16SrRNA genes, respectively. We also used another tool MapMi which (Ernoult et al., 2012) resulted in alignment of 744 unique sequences to mitochondrial DNA.

7.2 Nuclear encoded miR-4485 translocates to mitochondria

In eukaryotic cells mitochondria are known to import nuclear-encoded cytosolic RNAs (Sripada et al., 2012), although the transport of miRNAs to mitochondria is poorly studied. Previously we identified miR-4485 as one of the human miRNAs associated with mitochondria (Galimov et al., 2012). A sequence analysis showed that miR-4485 maps to

the intronic region of the MTRNR2L8 gene on chromosome 11 (Figure 7.2A) and its sequence aligns within the 16S rRNA region of the mitochondrial genome (Figure 7.2B).

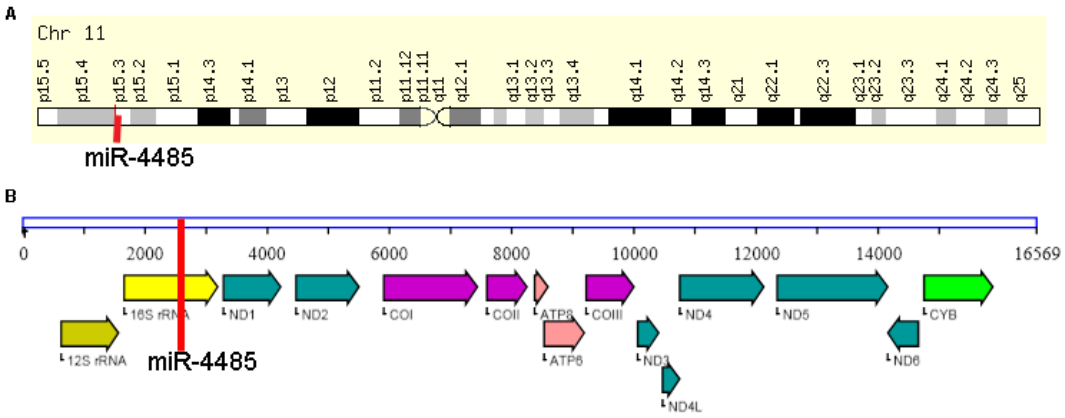

Figure 7.2.Genomic localization of miR-4485. *(A) The genomic locus of miR-4485 was determined by sequence mapping in UCSC genome browser and labeled on chromosome 11. (B) The linear graph of mitochondrial DNA sequence was plotted using DNA Dynamo v.1.41 and sequence homologous to mature miR-4485 was mapped.*

We hypothesized that the nuclear genome-encoded miR-4485 may translocate to mitochondria and regulate their functions. Levels of miR-4485 in mitochondria were analyzed by subcellular fractionation and quantitative real time PCR (qPCR). The levels of miR-4485 were high in the mitochondrial fraction, comparable to those of the 12S rRNA (Figure 7.3A). In our previous study we found that miR-145 localizes predominantly to the cytoplasm and is barely detectable in the mitochondrial fraction of HEK293 cells. Therefore, we used hsa-miR-145 (Zhang et al., 2014) as a negative control for mitochondrial association (Figure 7.3A). RNaseA treatment of purified mitochondria did not eliminate the association of miR-4485 (Figure 7.3B) indicating that the association is not mediated by RNA binding proteins located at the mitochondrial outer membrane. We also detected high levels of miR-4485 in mitoplasts (Figure 7.3C). The results indicate that miR-4485 is present inside mitochondria. In the cytosol pre-miRNAs are processed by Dicer to form the mature miRNA (Li et al., 2014). Previous studies demonstrated an enrichment of precursor miRNAs in the mitochondrial fraction (pre-mir-302, pre-let-7b, pre-mir-338) and their local processing to mature miRNAs (Lal et al., 2011; Feng et al., 2012). Our results show high levels of precursor miR-4485 in the cytosol (Figure 7.3D)

but low levels in mitochondria, suggesting that the precursor miR-4485 is present in the cytosol, while its mature form associates with mitochondria.

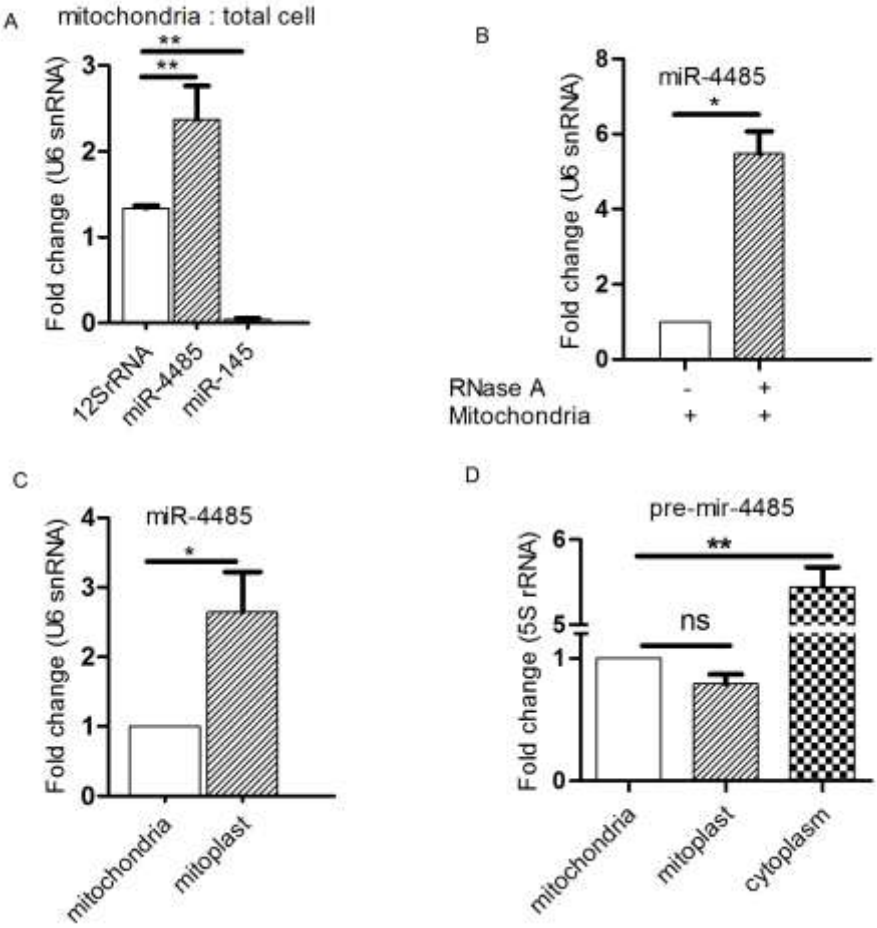

Figure 7 .3. miR-4485 associates with human mitochondria. *(A) Small RNA was extracted from total cell and mitochondria of HEK293, polyA tailing was performed and cDNA was synthesized. The relative association of 12S rRNA, miR-4485 and miR-145 was determined using U6 snRNA as endogenous control by qPCR. (B) Mitochondria were isolated from HEK293 and treated with RNaseA to remove non-specific bound RNA with mitochondria. The RNA was isolated and relative quantification of miR-4485 was performed by qPCR. (C) The mitoplasts were prepared from HEK293 and RNA extracted. The relative quantification was performed using U6 snRNA as endogenous control. (D) The purified fractions of mitochondria, mitoplast and cytosol were prepared from HEK293. The levels of pre-miR-4485 were determined in these fractions by qPCR.* $^{*}P < 0.01$; $^{**}P < 0.001$.

7.3 Nuclear transport and processing is required for the translocation of miR-4485 to mitochondria

As miR-4485 aligns to the nuclear as well as the mitochondrial genome we further investigated its origin and functional destination. To confirm that miR-4485 is encoded by mitochondrial DNA, we used mitochondrial DNA depleted ρ0 HeLa cells, which was obtained after passaging the cells in the presence of ethidium bromide (Hommers et al., 2012) (Mendell et al., 2012). The levels of mitochondrial DNA encoded ND4 and CYB gene transcripts were significantly decreased in ρ0 cells (Figure 7.4A). However levels of miR-4485 decreased in ρ0 cells, as compared to control cells (Figure 7.4B). As the genomic locus of miR-4485 maps to chromosome 11, the nuclear origin of miR-4485 was tested by inhibiting the nucleo-cytoplasmic translocation. The cells were treated with Leptomycin B, an inhibitor of CRM1 (exportin 1) involved in cytoplasmic translocation of RNAs (including miRNAs) and proteins from the nucleus (Bianchessi et al., 2015).

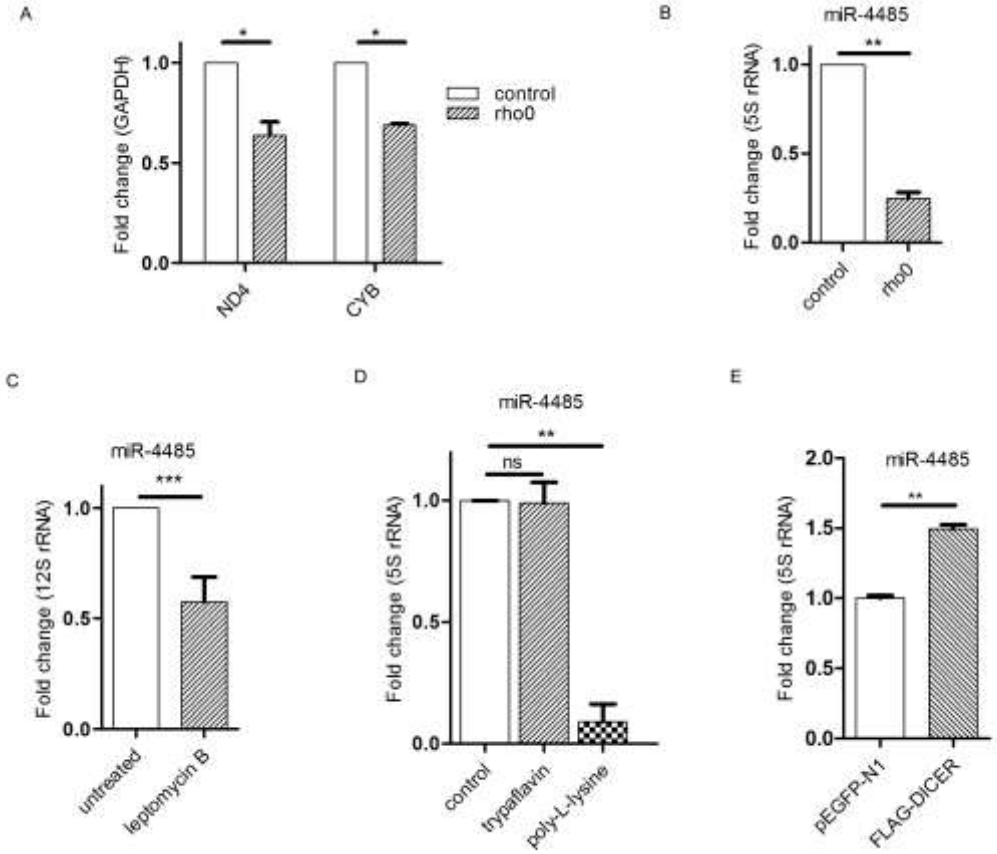

Figure 7.4. Nuclear transport and processing is required for association of miR-4485 with mitochondria. *(A) The levels of mitochondrial DNA encoded transcripts, ND4 and CYB were analyzed in control vsrho0 cells by qPCR. (B) The relative quantification of miR-4485 was performed by qPCR in same sets. (C) HEK293 cells were treated with nuclear exportin-1 inhibitor, leptomycin B. The RNA was extracted and the levels of miR-4485 were determined by qPCR. (D) HEK293 cells were treated with trypaflavin and Poly-L-Lysine. ThelevelofmiR-4485 was determined by qPCR.(E) HEK293 cells were transfected with pEGFP-N1 and Flag-Dicer and levels of miR-4485 was determined* $^{*}P < 0.01$, $^{**}P < 0.001$, $^{***}P < 0.0001$.

7.4 miR-4485 modulates the processing of mitochondrial genome encoded transcripts

To further analyze the role of miR-4485 in regulation of mitochondrial function and cell viability, we modulated miR-4485 activity by transfecting predesigned chemically modified RNA molecules that either mimic or inhibit the endogenous miRNA. We found an enrichment of miR-4485 in mitochondria fractions as compared to cytosolic fractions in the cells transfected with mimic. The cationic cyanine dyes tend to accumulate in mitochondria due to the high electrical membrane potential across the mitochondrial membrane (King et al., 1996). We used Cy5 tagged miR-4485 to increase its affinity to mitochondria and found that Cy5-miR-4485 levels were further increased in the mitochondrial fraction. However, the levels of 12S rRNA in both mimic and Cy5-miR-4485 transfected cells remained unchanged (Figure 7.6A) suggesting a specific enrichment of miR-4485.

As miR-4485 aligns within the 16S rRNA region in the mitochondrial genome, we hypothesized that it may affect levels of 16S rRNA and processing of the downstream mtDNA encoded transcripts. The processing of mitochondrial transcripts was analyzed in miR-4485 transfected cells by PCR amplifying RNA regions corresponding to pairs of adjacent transcripts (Figure 7.5A). In control cells, a 2353 bp PCR product representing the 16S rRNA and ND1 genes was observed, which corresponds to a processing intermediate of the 16S rRNA and ND1 pre-mRNA. In cells transfected with miR-4485 mimic, the intermediate form was enriched. Levels of precursor transcripts corresponding to the pair of genes located downstream to ND1 (ND1-ND2, ND2-COI, ATP6-COIII and COIII-ND3) showed similar levels in the control and miR-4485 mimic transfected cells,

while the transfection of miR-4485 mimic resulted in a down regulation of all mature mRNAs (Figure 7.5B). A direct binding of miR-4485 to mitochondrial 16S rRNA was confirmed by RNA immunoprecipitation using biotin-labelled-miR-4485

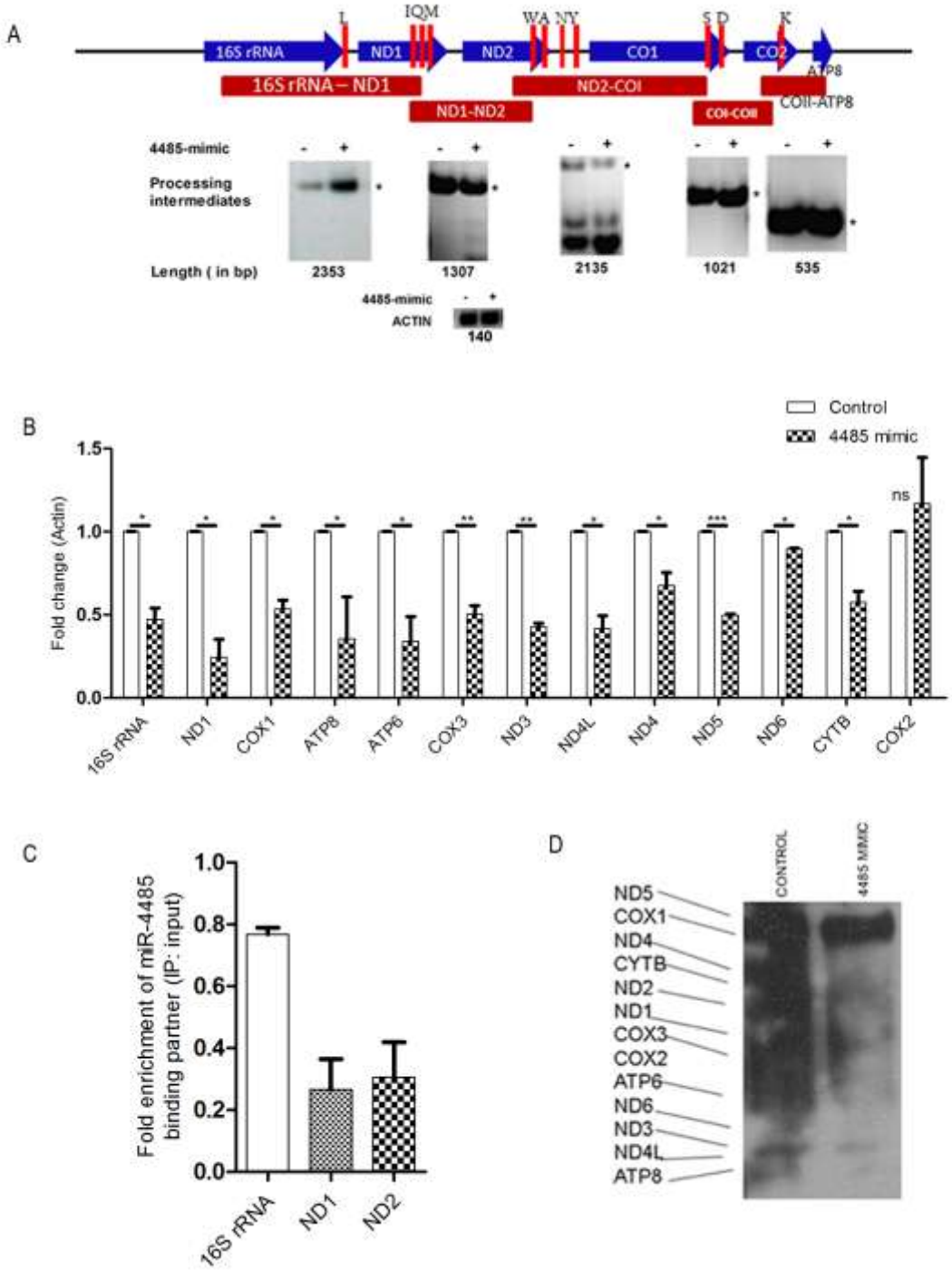

Figure 7.5. miR-4485 regulates mitochondrial RNA processing and translation. *(A) The role of miR-4485 on mitochondrial processing was analyzed as described in methods. Locations of mitochondrial DNA encoded specific transcripts (blue arrows) and tRNAs (pink marks) have been shown. Positions of the PCR*

amplicons were analyzed have been mapped on linear mitochondrial DNA map (red blocks). Gel images of processing intermediates in control and miR-4485 transfected HEK293 cells have been shown below the appropriate regions. The gel image of Actin was included as control. (B)Levels of mature mitochondrial genome encoded transcripts were determined by qPCR in HEK293 cells. (C) The biotin tagged miR-4485 was transfected and RNA-IP was performed as described in SI methods. The levels of 16S rRNA and mitochondrial DNA encoded transcripts was determined by qPCR in HEK293 cells. (D) The effect of miR-4485 on mitochondrial protein synthesis was determined by AHA incorporation and capture by biotin using Click-iT Labeling kit (Thermo-Fisher) in MCF-7 cells. $^{*}P < 0.01$, $^{***}P < 0.0001$.

16S rRNA was enriched in streptavidin beads, as compared to the other mitochondrial DNA transcribed transcripts like ND1 and ND2, which maps downstream of 16S rRNA (Figure 7.5C). miR-4485 interferes with the biogenesis of mitochondrial 16S rRNA and it may also affect the translation of mitochondrial DNA encoded proteins. Therefore, we monitored the level mitochondrial DNA encoded proteins by incorporation of the methionine homolog L-azidohomoalanine (AHA), followed by conjugation with biotin (Castanotto et al., 2009). The expression of miR-4485 decreased the level of all proteins encoded by the mitochondrial genome (Figure 7.5D). The results suggest that miR-4485 interferes with 16S rRNA processing and decreases mitochondrial protein synthesis.

7.5 miR-4485 regulates mitochondrial functions

As the mitochondrial genome encodes subunits of respiratory complexes, the down regulation of transcripts by miR-4485 may affect bioenergetic functions. The mitochondrial functions were analyzed in cells transfected with miR-4485 mimic and cy5-miR-4485. Indeed, both cellular (Figure 7.6B, Figure 7.7A) and mitochondrial (Figure 7.7B) ATP levels decreased in cells transfected with miR-4485 mimic. The transfection of miR-4485 mimic decreased the activity of mitochondrial respiratory complex-I (Figure 7.7C). Complex-I dysfunction is known to be associated with the increased production of ROS (Rhee et al., 2010). Interestingly, we observed both an elevated ROS level (Figure 7.7D, Figure 7.6C) and a decreased mitochondrial membrane potential (Figure 7.7E, Figure 7.6D) in miR-4485 mimic transfected cells, as compared to the control. Alterations in mitochondrial membrane potential and increased ROS are known to activate the

intrinsic cell death pathway (Tan et al., 2015). The transfection of miR-4485 mimic decreased the cell viability (Figure 7.7F) and increased the caspases3/7 activity (Figure 7.7G), which is characteristic of apoptosis induced by mitochondrial dysfunction (Murphy et al., 2009).The effects were more pronounced in Cy5-miR-4485 transfected cells. In contrast, the transfection of miR-4485 inhibitor increased the activity of complex-I (Figure 7.8A), ROS levels (Figure 7.8B) and improved mitochondrial membrane potential (Figure 7.8B). The inhibitor decreased caspase 3/7 activity (Figure 7.8C) and rescued cell death (Figure 7.8D).

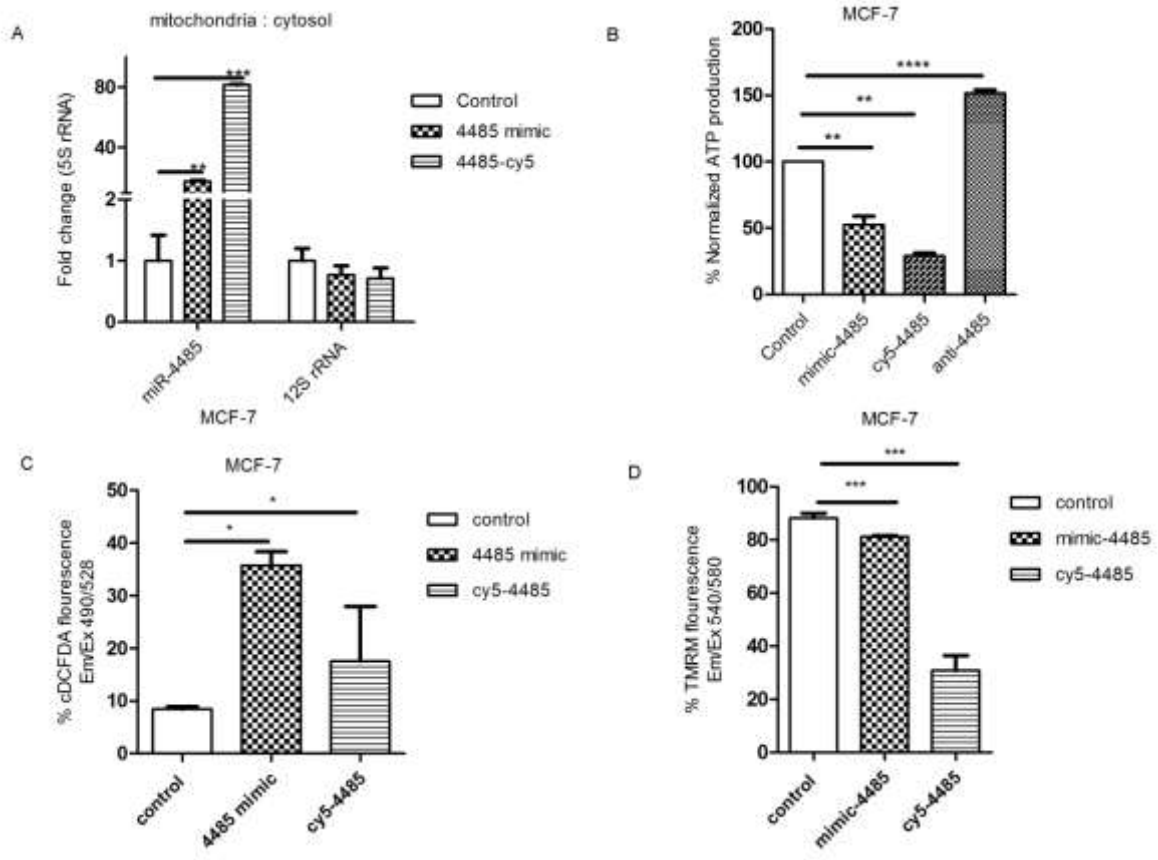

Figure 7.6. miR-4485 regulates mitochondrial functions in MCF-7. *(A) The association of miR-4485 with mitochondria was determined after transfection of its mimic or cy5 tagged (mitochondrial targeted) miR-4485 by fractionation followed by qPCR in MCF-7 cells. The mimic of miR-4485 was transfected along with control in MCF-7 cells and effect on mitochondrial functions was analyzed. (B) Levels of total cellular ATP were determined in using ATP detection kit. (C) The total cellular ROS produced was quantified by staining with CM-H_2DCFDA fluorescence using flourimeter and (D) Mitochondrial membrane potential by pre-incubating cells with TMRM in cells transfected with miR-4485.*

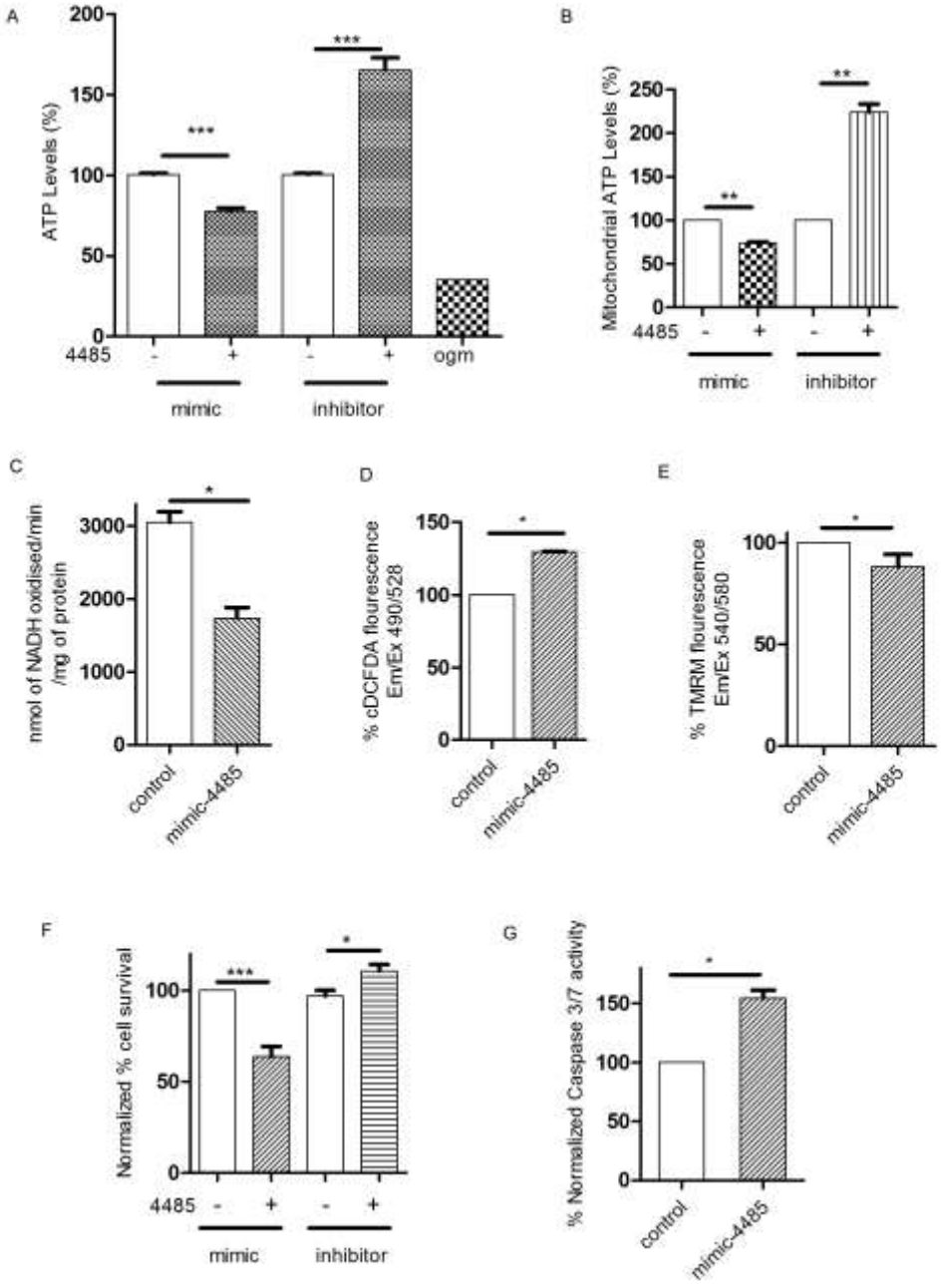

Figure 7.7. miR-4485 regulates mitochondrial function, ROS and cell death. *The mimic of miR-4485 was transfected along with control in HEK293 and effect on mitochondrial functions was analyzed. (A) Levels of total cellular ATP (oligomycin was added as control) and (B) mitochondrial ATP were measured using ATP detection kit. (C) The mitochondrial complex-I activity was measured as reduction of NADH per min/mg protein at 340 nm. (D) Total cellular ROS produced was quantified by staining with CM-H_2DCFDA fluorescence using flourimeter and (E) Mitochondrial membrane potential by pre-incubating cells with*

TMRM. (F) The cellular viability was determined by MTT assay while (G) Caspase 3/7 activity was determined using Caspase-GloR 3/7 Assay kit. $^{*}P < 0.01$, $^{***}P < 0.0001$.

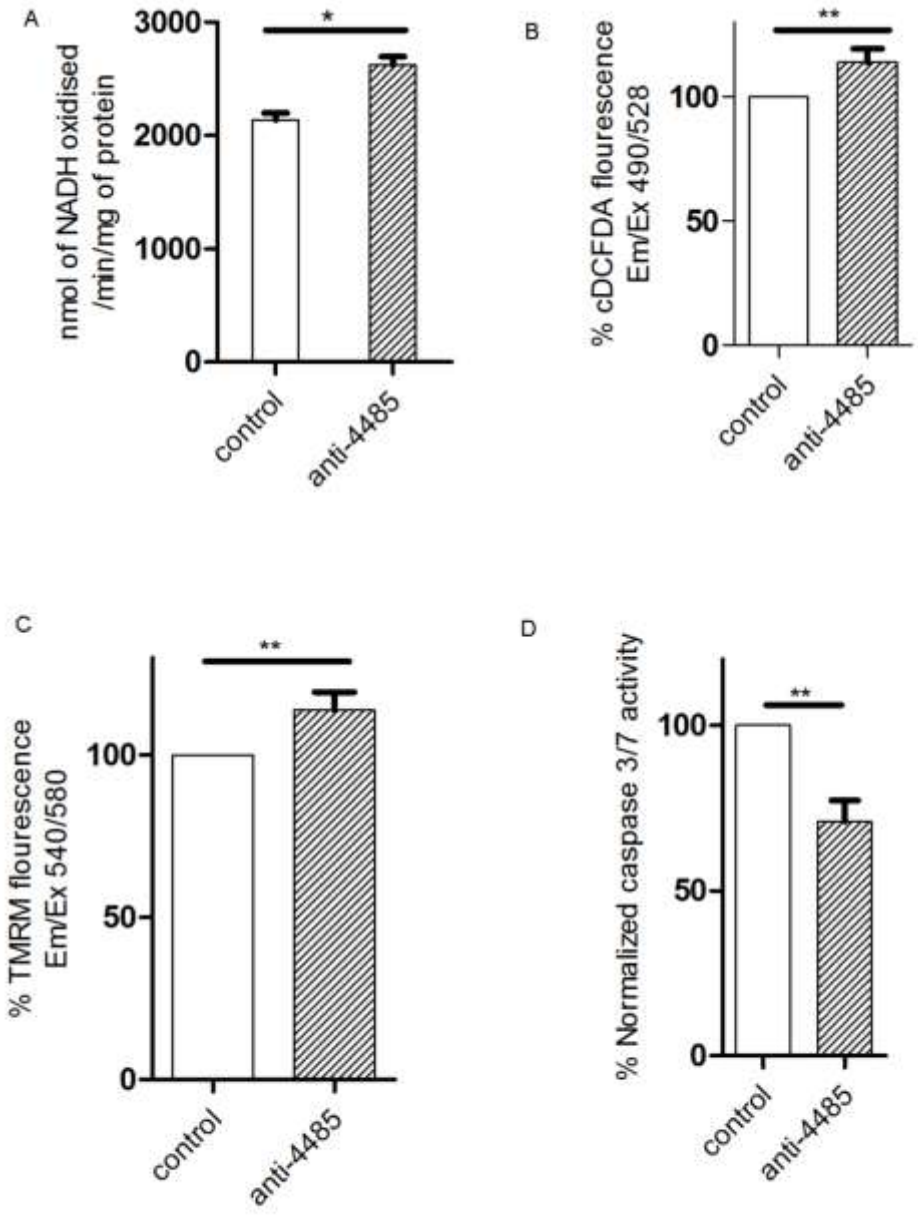

Figure 7.8. Inhibition of miR-4485 regulates mitochondrial functions. *The inhibitor of miR-4485 was transfected along with control in HEK293 and downstream effect on mitochondrial functions was analyzed by measuring (A) mitochondrial complex-I activity. (B) ROS levels. (C) The mitochondrial membrane potential and (D) Caspase3/7 activity.*

7.6 miR-4485 negatively affects the tumorigenic potential of breast cancer cells

To further understand pathophysiological implication of miR-4485 we looked for potential targets of miR-4485 by using StarBase v.2.0 tool, which provides high-confidence target prediction. Interestingly, most of the identified putative targets of miR-4485 had been implicated at various steps of tumorigenesis. The targets correspond to pathways involved in tumor suppression, tumor associated ligand shedding, metabolic reprogramming, migration of cancer cells and cell cycle control.

To validate the predictions, we tested the influence of miR-4485 mimic and inhibitor on levels of the most confidently predicted potential target transcripts. In the presence of the mimic, target transcript levels of five of the genes (NUP50, MDM4, STAT5B, MEIS2 and WDFY3) were decreased (Figure 7.9A) while the opposite was observed in the presence of miR-4485 inhibitor (Figure 7.9B). Alterations of mitochondrial functions and reprogramming of energy metabolism represent hallmarks of cancer (Bisroy et al., 2014; Xu et al., 2015). Aerobic glycolysis plays an essential role in the bioenergetics of cancer cells (Sullivan et al., 2014). To further understand the role of miR-4485 in tumorigenesis, we monitored markers of the glycolytic shift, lactate dehydrogenase (LDH) activity and clonogenic potential (shown as normalized plating efficiency) of breast carcinoma cells. Transcript levels of key glycolytic enzymes hexokinase 2 (HK2), pyruvate kinase 2 (PKM2) and phosphoglycerate mutase 1 (PGAM1) decreased in miR-4485 mimic transfected MCF-7 cells (Figure 7.10A) as compared to control. Further, transfection of miR-4485 mimic and Cy5-miR-4485 decreased the LDH activity (Figure 7.11A). The transfection of miR-4485 mimic in MCF-7 and MDA-MB-231 breast cancer cell lines decreased the clonogenic potential (Figure 7.10B, Figure 7.11B, Figure 7.11C).The transfection of miR-4485 inhibitor in MCF-7 had an opposite effect (Figure 7.11D). As we observed here miR-4485 regulates ROS levels (Figure 7.8D, Figure 7.9C), hence we analyzed the clonogenic potential of breast cancer cells in the presence of ROS scavenger N-acetyl-L-cysteine (NAC). Cell survival (Figure 7.10C) and plating efficiency (Figure 7.10D) significantly increased in miR-4485 mimic transfected MCF-7 cells in the presence of NAC. Similar results were observed in the cervical carcinoma cell line, HeLa (Figure

7.11D). These results suggest that miR-4485 may down regulate glycolysis and decrease clonogenic potential of breast cancer cells.

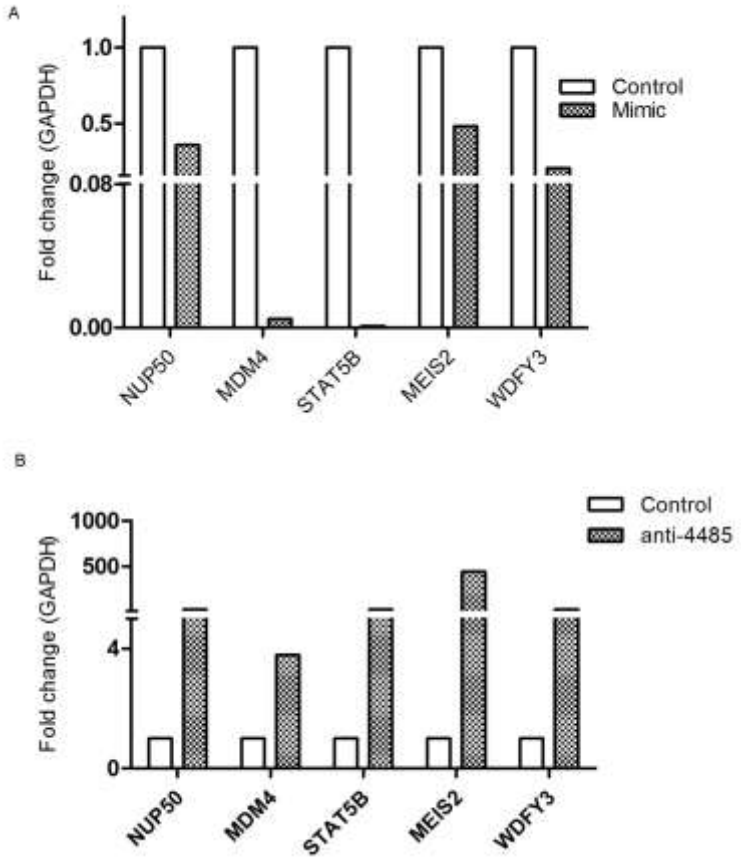

Figure 7.9. miR-4485 expression alters putative target transcripts. *The putative targets were determined from starBasev.2.0. The relative expression of target transcripts was determined by qPCR in MCF-7cells transfected with miR-4485 mimic (A) and inhibitor (B) (n=3).*

We further tested the role of miR-4485 as tumor suppressor in a xenograft model. Cells corresponding to a stable clone of triple-negative breast carcinoma MDA-MB231 cells carrying amiR-4485 expressing construct were inoculated subcutaneously into nude mice and the tumorigenicity was compared against the control cells carrying empty vector. Tumorigenicity of miR-4485 overexpressing cells was reduced. The progression of tumor growth was delayed with a remarkable decline of 25-62% in tumor volume (Figure 7.11E). We then analyzed the expression of miR-4485 in breast cancer patients. Levels of both

mature miR-4485 and its precursor transcript were found decreased in 7 breast tumor tissues, as compared to the non-tumoral adjacent tissue (NAT) (Figure 7.11F). These results indicate that mitochondrial targeted miR-4485 negatively regulates tumorigenicity in breast cancer.

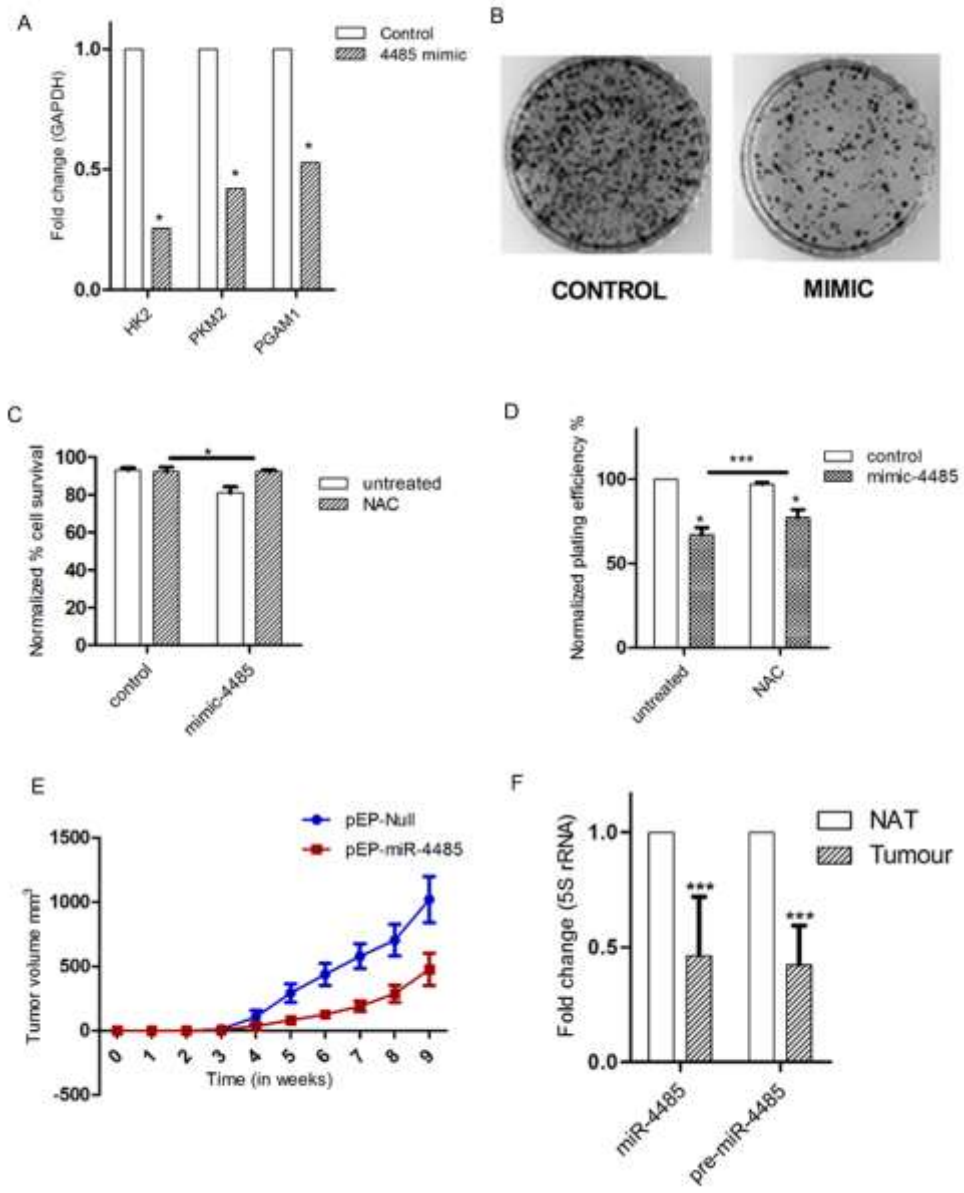

Figure 7.10. miR-4485 negatively regulates tumorigenic potential of breast cancer cells. *(A) The total cellular RNA was prepared from MCF-7 cells transfected with mimic. The relative expression of the genes regulating glycolysis was analyzed by real time PCR. (B) The mimic of miR-4485 was transfected in MCF-7 cells and clonogenic assay was performed. To further elucidate the role of ROS in tumorigenesis, MCF-7 was transfected with control and miR-4485 mimic, thereafter treated with NAC. The cell viability by MTT*

assay (C) and plating efficiency by clonogenic assay (D) were analyzed. (E) miR-4485 expression decreased tumorigenic potential of transformed cells in vivo. Average volume was plotted with ±SEM of values (n=24) and p value 0.0154. (F) The expression pattern of miR-4485 in tumor tissue was determined qPCR (n=7). $^{*}P < 0.01,^{***}P < 0.0001$.

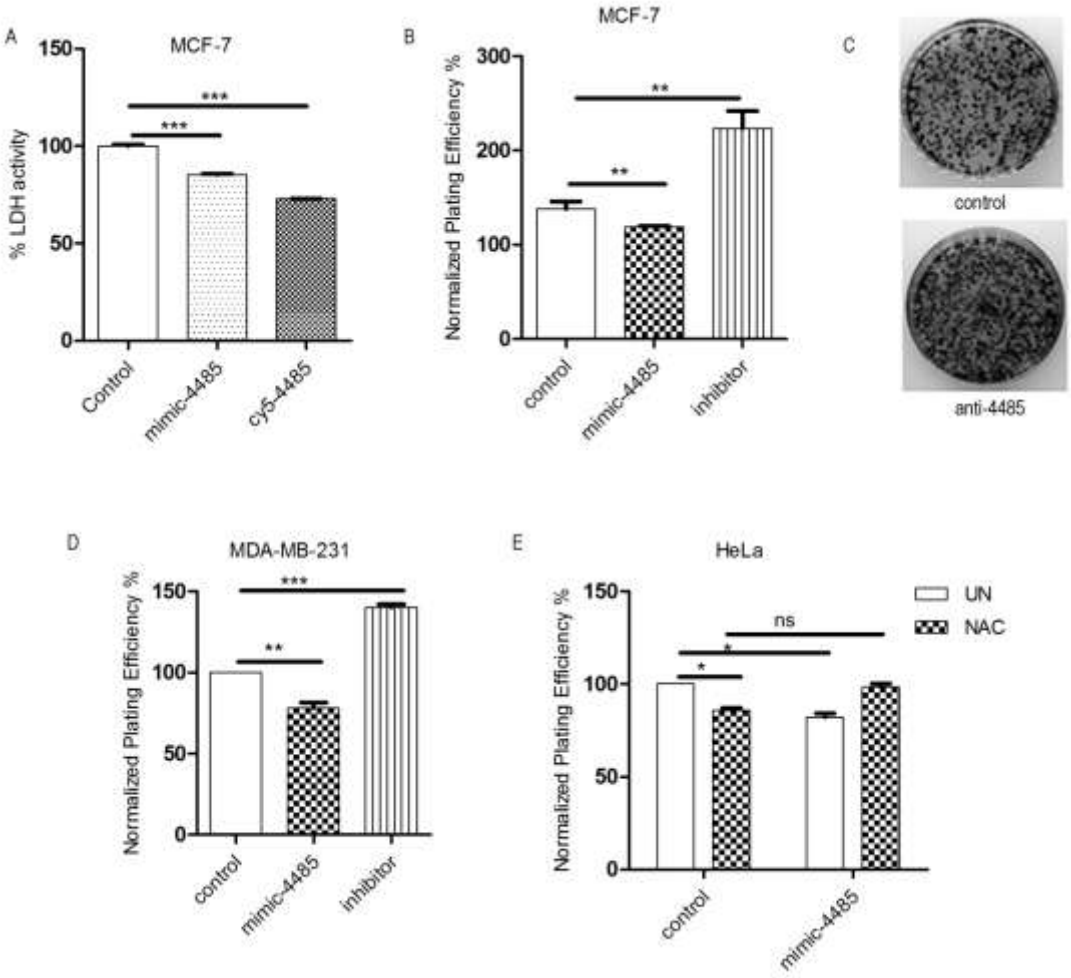

Figure 7 .11. Inhibition of miR-4485 increases tumorigenic potential. *(A) The LDH content in the cells transfected with miR-4485 and cy5-4485 was determined by colorimetric assay from cell lysates as mention in methods. The mimic (B) and inhibitor (C) of miR-4485 was transfected and plating efficiency was determined inMCF-7. (D) MDA-MB-231 cells were transfected with mimic, anti-miR-4485 and controls and plating efficiency determined as described in Figure 4. (E) HeLa cells were transfected with miR-4485 mimic to perform colony forming assay in the presence of ROS scavenger, NAC.*

7.7 Discussion

The regulation of mitochondrial function is critical for maintaining cellular and tissue homeostasis. The mitochondria are endosymbiotic organelles with a separate circular DNA genome encoding a limited number of mitochondrial proteins, whereas the majority of mitochondrial proteins are encoded by the nuclear genome. The replication of the mitochondrial genome, the transcription and expression of its transcripts is co-ordinated by nuclear genome encoded proteins and RNA that are imported to mitochondria (Webster et al., 2012). Previous reports from our group (Zhang et al., 2014) had identified a set of miRNAs associated with mitochondria, although their potential significance under normal and stress conditions is not yet understood. Here we show that miR-4485 is transcribed from the nuclear genome, then translocates to mitochondria and affects expression and/or processing of mitochondrial transcripts thereby contributing to metabolic regulation and tumor suppression.

Analysis of the human genome suggests that miR-4485 may originate from the intronic region of MTRNR2L8 gene on chromosome 11. miR-4485 levels were enriched in the RNaseA treated mitochondria and mitoplasts suggesting its specific localization inside mitochondria. Recently it was also suggested that miR-4485 may represent a processing product of ASncmtRNA-2, a lncRNA derived from the 16S rRNA region of the mitochondrial genome (Ueda et al., 2002) hence we cannot rule out the possibility that a sub-fraction of miR-4485 originates from the mitochondrial genome. Here we show that both mitochondrial DNA depletion and the inhibition of nuclear transport decreased the level of miR-4485 in mitochondria, and miR-4485 precursor transcript was enriched in cytosol. The inhibition of Dicer activity decreased the level of miR-4485 in mitochondria. Also, the inhibition of TRBP-Ago2 interaction by trypaflavin did not affect the levels of miR-4485 in mitochondria further confirming that processing by Dicer is required. The results here indicate that miR-4485 is encoded by the nuclear genome, processed through the canonical miRNA pathway and translocates to mitochondria.

The translocation of miR-4485 to mitochondria may represent one of the mechanisms of nuclear control and fine tuning of mitochondrial functions. The levels of miR-4485 in mitochondria were altered under different conditions suggesting a stimuli specific import.

Further, we studied the significance of translocation of miR-4485 to mitochondria. Transfection of miR-4485 mimic showed decreased levels of mitochondrial transcripts. The mitochondrial genome transcribes a polycistronic as RNA precursor, which is further processed to mature mRNAs by a rather complex process. Recently, 107 nuclear encoded mitochondrial proteins with RNA-binding properties were screened to identify new regulatory factors required for the maturation of mitochondrial transcripts (Wolf et al., 2014; Jourdain et al., 2016; Popow et al., 2015; Antonicka H, et al., 2015). The results here suggest that a nuclear encoded miR-4485 may also affect the biogenesis of mitochondrial DNA encoded transcripts, particularly the transcript corresponding to the 16S rRNA gene. We suggest that miR-4485 may either bind to the 16S rRNA region of mtDNA to affect its transcription, or it may directly bind to 16S rRNA and inhibit its processing. We found that the binding of miR-4485 to the 16S rRNA/ND1 mRNA precursor transcript may inhibit the processing leading to an accumulation of unprocessed transcripts (Jourdain et al., 2016; Popow et al., 2015; Antonicka H, et al., 2015). This provides an additional mechanism for ensuring proper OXPHOS complex stoichiometry. Our study is further supported by a recent report showing that a nuclear encoded miR-1 is also processed in the cytoplasm, transported to mitochondria where it regulates mitochondrial transcripts (Calvo et al., 2010) specifically in muscle cells. It will be interesting to further investigate the processing of miR-4485 and translocation to mitochondria in cells with different bioenergetic requirements to understand the role of nuclear encoded miRNAs in the fine-tuning of mitochondrial functions.

Mitochondrial complex-I is the largest subunit of the mitochondrial electron transport chain. As the entry point for electrons from NADH it is important for the maintenance of the NAD/NADH ratio. The multi-subunit complex-I is composed of nuclear and mitochondrial genome encoded proteins. The mitochondrial encoded subunit forms the core of complex-I (Stroud et al., 2016). Indeed, the miR-4485 mimic affects mitochondrial transcripts, which down regulate mitochondrial respiratory complex-I activity and the level of mitochondrial ATP. The compromised complex-I activity may reverse the flow of electrons to molecular oxygen (Halestrap et al., 1982) leading to the observed elevation of ROS, decreased mitochondrial membrane potential and activation of the intrinsic cell death pathway. Given the role of miR-4485 in regulating the processing of mitochondrial

transcripts, miR-4485 may be also implicated in the array of genetic, developmental and metabolic disorders.

A reprogramming of mitochondrial bioenergetics is one of the important events leading to cancer progression. The modulation of mitochondrial complex-I activity and ROS level by miR-4485 suggests its participation in metabolic reprogramming of cancer cells (Oronsky et al., 2014; Xu et al., 2012; Sullivan et al., 2012). Elevated ROS may have differential effects in cancer and act as double-edged sword. Both reduced and high levels of ROS are known to activate cell death pathways (Chenung et al., 2016; Weinberget al., 2010; Chandel et al., 1998). However, the detailed mechanism of miR-4485 induced ROS mediated regulation and its role in tumorigenesis needs to be further studied. We found that the miR-4485 mimic decreased the levels of transcripts for key enzymes of the glycolytic pathway; however these are not the direct targets of miR-4485. Cancer cells specifically rely on the aerobic glycolysis (also known as the Warburg effect) as major source of energy and anaplerotic replenishing TCA cycle with intermediate metabolites for rapid proliferation (Weinberg et al., 2015). The decrease in transcript levels of glycolytic enzymes and mitochondrial complex-I activity in the presence of miR-4485 could negatively affect cancer cell metabolism. In agreement, we observed decreased clonogenic ability of breast cancer cells in vitro and decreased tumorigenicity in a nude mice xenograft model. Interestingly, a decreased expression of miR-4485 was observed in tumor tissue, as compared to control tissue samples from breast cancer patients, further suggesting its anti-tumor effects. It would be important to understand the dynamics of miR-4485 expression and translocation to mitochondria at different stages of cancer progression, along with changes in mitochondrial functions in different models of cancer. However, levels and activity of miR-4485 in the cytosol cannot be neglected and needs to be further studied to learn more about nucleo-mitochondrial crosstalk via miRNA. This may have important implication in understanding the role of nuclear encoded miRNAs in metabolic reprogramming not only in breast cancer but also in tumors of different origin and in metabolic disorders.