Free Radical Biology and Medicine 89 (2015) 1036–1048

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original contribution

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

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ARTICLE INFO

Article history:
Received 17 May 2015
Received in revised form
27 October 2015
Accepted 28 October 2015
Available online 31 October 2015

Keywords:
Ubiquitin E3 ligase
TRIM4
Mitochondria
Cell death
H_2O_2
PRX1

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

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http://dx.doi.org/10.1016/j.freeradbiomed.2015.10.425
0891-5849/

Biochimica et Biophysica Acta 1853 (2015) 1073–1086

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

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

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ARTICLE INFO

Article history:
Received 24 September 2014
Received in revised form 23 December 2014
Accepted 21 January 2015
Available online 30 January 2015

Keywords:
NLRX1
Caspase-8
Mitochondria
Metabolic reprogramming
Inflammation
Cancer

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

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http://dx.doi.org/10.1016/j.bbamcr.2015.01.016
0167-4889/

Biochimica et Biophysica Acta 1852 (2015) 451–461

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

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

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ARTICLE INFO

Article history:
Received 1 September 2014
Received in revised form 22 November 2014
Accepted 26 November 2014
Available online 4 December 2014

Keywords:
TNF-α
Inflammation
Parkinson's disease
Mitochondria
miRNA

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

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http://dx.doi.org/10.1016/j.bbadis.2014.11.019
0925-4439/

Mitochondrion 16 (2014) 50–54

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

Side effects of antibiotics during bacterial infection: Mitochondria, the main target in host cell

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ARTICLE INFO

Available online 16 November 2013

Keywords:
Antibiotics
Mitochondria
Side effects
Translational arrest

ABSTRACT

Antibiotics are frontline therapy against microbial infectious diseases. Many antibiotics are known to cause several side effects in humans. Ribosomal RNA (rRNA) is the main target of antibiotics that inhibit protein synthesis. According to the endosymbiont theory, mitochondrion is of bacterial origin and their molecular and structural components of the protein expression system are almost similar. It has been observed that the rate of mutations in mitochondrial rRNA is higher as compared to that of nuclear rRNA. The presence of these mutations may mimic prokaryotic rRNA structure and bind to antibiotics targeted to ribosomes of bacteria. Mitochondrial functions are compromised hence may be one of the major causes of side effects observed during antibiotic therapy. The current review had summarized the studies on the role of antibiotics on mitochondrial functions and its relevance to the observed side effects in physiological and pathological conditions.

1. Introduction

Since the development of antibiotics, it had been the first line of treatment against many Gram positive and negative bacterial infections since the early twentieth century. The compounds that specifically target fundamental cellular processes of bacteria, with negative consequences for pathogen survival, and no plausible side effects in host are considered as potentially useful antibiotics (Dimauro and Davidzon, 2005; Fischel-Ghodsian, 2005; Wang et al., 2006). It has been observed that more than 40% of antibiotics interfere with bacterial protein biosynthesis machinery and more specifically the ribosome is one of the most important targets (McCoy et al., 2011). The two ribosomal subunits (30S and 50S) play an important role starting from initiation of elongation to termination of the translational process. The 30S subunit is responsible for codon–anticodon interactions. In the prokaryotic system, the translation is a complex process and involves several steps like initiation, elongation and termination which had been well established. The initiation of the translational is initiated by a complex of IF1, IF2 (a GTP-binding protein), IF3, mRNA and the initiator fMet-tRNA^{fMet} which binds to the 30S ribosomal subunit, forming the 30S initiation complex (30S IC). In the next step, the 50S subunit joins the 30S IC and GTP is hydrolyzed. This leads to the disassociation of the initiation factors and fMet-tRNA^{fMet} is positioned in the P site. This complex is called as 70S initiation complex (70S IC). After binding of the first aminoacyl-tRNA and formation of the first peptide bond, the 70S IC enters the elongation cycle of translation and finally termination. The 50S subunit is responsible for the catalytic activity of the peptide bond formation. It had been observed that different steps of translational process are the direct target of different antibiotics. The mode of action at different steps of translation had been summarized in Table S1.

The peptidyl transferase center (PTC) is the most conserved rRNA nucleotide in the entire ribosome. The recent findings revealed an unexpected high level of variation in the mode of antibiotic binding to the PTC of ribosomes from different organisms (Polacek and Mankin, 2005). The antibiotics chloramphenicol and oxazolidinones act on the PTC and cause myelosuppression, lactic acidosis and optic and peripheral neuropathies similar to phenotypes frequently found in mitochondriopathies (Bacino et al., 1995; Bitner-Glindzicz et al., 2010). Similarly, another group of antibiotics, erythromycin, tetracycline and aminoglycosides, which target large and small subunits of rRNA also showed similar side effects (Pasquale and Tan, 2005). These are also known to act on mitochondria and may cause bioenergetic crisis in a patient with Leber's hereditary optic neuropathy (LHON) disease (Thyagarajan et al., 2000). Mitochondria are now known to be involved in many other cellular processes other than energy metabolism like apoptosis and regulation of inflammation during viral and bacterial infections (Zhao et al., 2004). However, the action of antibiotics in the context of the newly discovered role of mitochondria has not been studied. The antibiotics had been extensively used as front-line therapy against many bacterial infections and are considered as life saving drugs. The side effects of

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http://dx.doi.org/10.1016/j.mito.2013.10.005

Biochimica et Biophysica Acta 1842 (2014) 144–153

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

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

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ARTICLE INFO

Article history:
Received 16 June 2013
Received in revised form 26 October 2013
Accepted 7 November 2013
Available online 13 November 2013

Keywords:
MITA
Tumor suppressor gene
NF-κB
Breast cancer

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 400,000 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

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http://dx.doi.org/10.1016/j.bbadis.2013.11.006

Biochimica et Biophysica Acta 1833 (2013) 3134–3144

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

TRIM13 regulates caspase-8 ubiquitination, translocation to autophagosomes and activation during ER stress induced cell death

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ARTICLE INFO

Article history:
Received 12 April 2013
Received in revised form 28 August 2013
Accepted 30 August 2013
Available online 8 September 2013

Keywords:
Endoplasmic reticulum stress
Autophagy
Cell death
Ubiquitin ligase
TRIM13
Caspase-8

ABSTRACT

The emerging evidences suggest that endoplasmic (ER) stress is involved in onset of many pathological conditions like cancer and neurodegeneration. The persistent ER stress results in misfolded protein aggregates, which are degraded through the process of autophagy or lead to cell death through activation of caspases. The regulation of crosstalk of autophagy and cell death during ER stress is emerging. Ubiquitination plays regulatory role in crosstalk of autophagy and cell death. In the current study, we describe the role of TRIM13, RING E3 ubiquitin ligase, in regulation of ER stress induced cell death. The expression of TRIM13 sensitizes cells to ER stress induced death. TRIM13 induced autophagy is essential for ER stress induced caspase activation and cell death. TRIM13 induces K63 linked poly-ubiquitination of caspase-8, which results in its stabilization and activation during ER stress. TRIM13 regulates translocation of caspase-8 to autophagosome and its fusion with lysosome during ER stress. This study first time demonstrated the role of TRIM13 as novel regulator of caspase-8 activation and cell death during ER stress.

1. Introduction

The endoplasmic reticulum (hereafter ER) is the primary site for synthesis and folding of proteins in eukaryotes. The accumulation of misfolded proteins and its aggregation leads to unfolded protein response (UPR) which prevents further protein burden and damage to the cell [1,2]. Unfolded proteins in the ER are generally tagged with ubiquitin and degraded by ubiquitin proteasome system (UPS), known as ER associated degradation system-I (ERAD-I) [3,4]. It has been observed that accumulation of misfolded proteins in ER may lead to formation of protein aggregates, which are toxic to cell. The process of autophagy, also known as ERAD-II/macroautophagy clears these protein aggregates [5,6]. Specialized sensors like IREα, PERK and ATF6, sense the persistent ER stress to maintain either ER homeostasis or initiate cell death pathways [1,7]. Recent evidences suggest that autophagy may be adaptive response during ER stress leading to either cell death or survival. The cross talk of autophagy and cell death during ER stress has implication in many chronic conditions like neurodegeneration, cancer and metabolic diseases [2,8,9]. Tumor cells have increased unfolded/misfolded proteins due to inadequate supply of glucose and subsequent reduction in glycosylation of proteins and ATP, which results in ER stress. The induction of autophagy during the ER stress plays critical role in tumor cell survival, thus contributing significantly to tumorigenesis. The regulators of cross talk between autophagy and cell death during ER stress are not well understood.

Increasing evidences suggest that ubiquitin mediated post-translational modification is the central mechanism for regulation of crosstalk between autophagy, cell death and survival [10,11]. Ubiquitination of target protein involves sequential action of three enzymes: E1, E2 and E3, for transferring the ubiquitin to the target protein [12]. The terminal enzyme E3, transfers Ub from the E2 to a lysine residue on a substrate protein, resulting in an isopeptide bond formation between the substrate lysine and the C-terminus glycine of Ub. E3 ligases provide specificity to the pathway as they recognize the substrate, interact with definite E2 and determine the topology of ubiquitination. Several ubiquitin E3 ligases have been identified which determine the critical pattern of substrate ubiquitination leading to unique outcome, either degradation of proteins through UPS or regulation of their activity [13,14]. The role of ubiquitin ligases regulating crosstalk of cell death and autophagy during ER has not been investigated in detail.

Ubiquitin E3 ligases have been broadly classified in three major families called as RING, HECT and U Box. The majority of the ubiquitin ligases belong to RING family which are characterized by the presence of RING domain [15]. The role of RING E3 ligases have not been well studied in the context of many different cellular functions. TRIM family proteins (>70), member of RING type ubiquitin E3 ligases, are characterized by the presence of N-terminal RING, B-Box, Coiled Coil (CC) domain and variable C-terminal domain [16]. The role of TRIM family proteins are emerging in several processes like innate immune response, regulation of

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0167-4889/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamcr.2013.08.021

OPEN ACCESS Freely available online

PLOS ONE

Nucleo-Cytoplasmic Trafficking of TRIM8, a Novel Oncogene, Is Involved in Positive Regulation of TNF Induced NF-κB Pathway

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Abstract

TNF induced nuclear factor kappa B (NF-κB) is one of the central signaling pathways that plays a critical role in carcinogenesis and inflammatory diseases. Post-translational modification through ubiquitin plays important role in the regulation of this pathway. In the current study, we investigated the role of TRIM8, member of RING family ubiquitin ligase in regulation of NF-κB pathway. We observed that TRIM8 positively regulates TNF induced NF-κB pathway. Different domains of TRIM8 showed discrete functions at the different steps in regulation of TNF induced NF-κB pathway. Ubiquitin ligase activity of TRIM8 is essential for regulation of NF-κB activation in both cytoplasm as well as nucleus. TRIM8 negates PIAS3 mediated negative repression of NF-κB at p65 by inducing translocation of PIAS3 from nucleus to cytoplasm as well as its turnover. TNF induces translocation of TRIM8 from nucleus to cytoplasm, which positively regulates NF-κB. The cytoplasmic translocation of TRIM8 is essential for TNF induced NF-κB but not for p65 mediated NF-κB regulation. TRIM8 also enhanced the clonogenic and migration ability of cells by modulating NF-κB. The further study will help to understand the role of TRIM8 in inflammation and cancer.

Citation: Tomar D, Sripada L, Prajapati P, Singh R, Singh AK, et al. (2012) Nucleo-Cytoplasmic Trafficking of TRIM8, a Novel Oncogene, Is Involved in Positive Regulation of TNF Induced NF-κB Pathway. PLoS ONE 7(11): e48662. doi:10.1371/journal.pone.0048662

Editor: Joao P. B. Viola, National Cancer Institute (INCA), Brazil

Received May 28, 2012; **Accepted** September 28, 2012; **Published** November 12, 2012

Funding: This study was supported by the Program Support to Indian Institute of Advanced Research (IIAR) sponsored by Department of Biotechnology (DBT), Govt. of India. Dhanendra Tomar received Senior Research Fellowship from Council of Scientific and Industrial Research (CSIR), Govt. of India. Lakshmi Sripada received Junior Research Fellowship from University Grant Commission (UGC), Govt. of India. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

NF-κB is an inducible transcription factor and known to be involved in various physiological and pathological conditions [1]. The activation of NF-κB leads to transcription of the genes regulating cell cycle, immune response and cell death [2,3,4]. The dysregulation of this pathway has been observed in many cancer, neurodegeneration, skeletal abnormalities, autoimmune diseases and metabolic disorders [5,6]. It is regulated by variety of patho-physiological stimuli however generates a unique response for particular stimuli. Tumor necrosis factor alpha (TNF-alpha) induced NF-κB affects many cellular functions including growth, differentiation, inflammation, immune responses and apoptosis through regulation of NF-κB pathway [7,8]. Hence, TNF induced NF-κB pathway has been a focus of investigation for last several years [1,2,3,6,9].

Posttranslational modification of proteins by ubiquitin has been known to play important role in regulation of NF-κB pathway. The process of ubiquitination is achieved by the sequential action of three enzymes: E1 (Ub activating enzyme), E2 (Ub conjugating enzyme), E3 (Ub ligases). Recent evidences suggest that all the enzymes of this pathway have unique role in regulation of NF-κB pathway [9]. The terminal enzyme E3, transfers Ub from the E2 to a lysine residue on a substrate protein, resulting in an isopeptide bond formation between the lysine of substrate and the C-terminal glycine of Ub. E3 ligases provide specificity to the pathway as they recognize the substrates, interact with definite E2 to determine the topology of ubiquitination. The role of ubiquitination in regulation of NF-κB pathway is evolving and several untraditional roles have been discovered like stabilization of proteins through K63 linkages, formation of linear ubiquitin (Ub) chains [9,10,11]. E3 ligases may be critical in many of these unique linkages of target proteins through ubiquitin.

The binding of TNF to its cognate receptor TNFR1, leads to recruitment of several ubiquitin ligases like TRAF2, cIAP1 and cIAP2 and kinase RIP1 [12]. These ligases are either auto-ubiquitinated and/or ubiquitinate other substrates to activate downstream central kinase complex (IKKα/IKKβ/IKKγ), resulting in translocation of NF-κB to nucleus. Interestingly, it has been recently observed that two proteins known as heme-oxidised IRP1 Ub ligase-1 (HOIL-1) and the HOIL-1-interacting protein (HOIP), which together constitute Linear Ubiquitin-chain Assembly complex (LUBAC) is recruited to TNFR1 in a ligand-dependent manner [13]. This complex forms linear ubiquitin chains that regulate activation of NF-κB [14]. These evidences suggest that there is additional level of complexity in regulation of NF-κB activation through recruitment of different ubiquitin ligases in cell type and stimulus specific conditions.

TRIM/RBCC belongs to subset of RING family of Ub E3 ligases, consisting of N terminus **R**ING domain, **B**-Box and coiled-coil (**CC**) domain (**RBCC**) [15]. TRIM proteins have been implicated in a variety of processes like development, differenti-

Article

Inhibition of Inositol 1, 4, 5-trisphosphate receptor induce breast cancer cell death through deregulated autophagy and cellular bioenergetics[†]

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Running title: IP_3R in breast cancer

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Keywords: Breast cancer; Autophagy; Calcium channels; Bioenergetics; Insositol 1,4,5 trisphosphate receptor

Additional Supporting Information may be found in the online version of this article.

Received 15 April 2016; Revised 18 January 2017; Accepted 18 January 2017
Journal of Cellular Biochemistry

DOI 10.1002/jcb.25891

OPEN ACCESS Freely available online

PLOS ONE

Systematic Analysis of Small RNAs Associated with Human Mitochondria by Deep Sequencing: Detailed Analysis of Mitochondrial Associated miRNA

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Abstract

Mitochondria are one of the central regulators of many cellular processes beyond its well established role in energy metabolism. The inter-organellar crosstalk is critical for the optimal function of mitochondria. Many nuclear encoded proteins and RNA are imported to mitochondria. The translocation of small RNA (sRNA) including miRNA to mitochondria and other sub-cellular organelle is still not clear. We characterized here sRNA including miRNA associated with human mitochondria by cellular fractionation and deep sequencing approach. Mitochondria were purified from HEK293 and HeLa cells for RNA isolation. The sRNA library was generated and sequenced using Illumina system. The analysis showed the presence of unique population of sRNA associated with mitochondria including miRNA. Putative novel miRNAs were characterized from unannotated sRNA sequences. The study showed the association of 428 known, 196 putative novel miRNAs to mitochondria of HEK293 and 327 known, 13 putative novel miRNAs to mitochondria of HeLa cells. The alignment of sRNA to mitochondrial genome was also studied. The targets were analyzed using DAVID to classify them in unique networks using GO and KEGG tools. Analysis of identified targets showed that miRNA associated with mitochondria regulates critical cellular processes like RNA turnover, apoptosis, cell cycle and nucleotide metabolism. The six miRNAs (counts >1000) associated with mitochondria of both HEK293 and HeLa were validated by RT-qPCR. To our knowledge, this is the first systematic study demonstrating the associations of sRNA including miRNA with mitochondria that may regulate site-specific turnover of target mRNA important for mitochondrial related functions.

Citation: Sripada L, Tomar D, Prajapati P, Singh R, Singh AK, et al. (2012) Systematic Analysis of Small RNAs Associated with Human Mitochondria by Deep Sequencing: Detailed Analysis of Mitochondrial Associated miRNA. PLoS ONE 7(9): e44873. doi:10.1371/journal.pone.0044873

Editor: Sebastien Pfeffer, French National Center for Scientific Research - Institut de biologie moléculaire et cellulaire, France

Received March 12, 2012; **Accepted** August 9, 2012; **Published** September 11, 2012

Funding: This work was supported by Department of Biotechnology, Govt. of India (BT/PR12906/AGR/36/638/2009). Dhanendra Tomar received Senior Research Fellowship from CSIR (Council of Scientific & Industrial Research), Govt. of India. Lakshmi Sripada received Junior Research Fellowship from UGC (University Grants Commission), Govt. of India. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Mitochondria are known for a long time as power house of cell and are primary site for various metabolic activities like respiration, fatty and amino acid metabolism [1,2,3]. Increasing evidences suggest its role in many other processes like cell death, inflammation and innate immune response [4,5,6]. The role of mitochondria is now known to be associated with wide range of pathological conditions like cancer, neurodegeneration, ageing, inflammation and infection [7,8,9]. The further study of mitochondrial functions and its regulation in various pathological conditions needs to be elucidated.

Human mitochondrial genome is 16.5 kb circular DNA and is known to encode 13 proteins subunits of mitochondrial respiratory complexes, 22 tRNA and 2 rRNAs [10,11]. The mitochondria are not completely autonomous and require nuclear encoded proteins for their optimal function. Interestingly, proteome of mitochondria from several species including human have been now well studied and is known to contain more than 1500 proteins [12]. The majority of proteins are encoded by nuclear DNA, translated in cytoplasm and imported to mitochondria. The translocation of proteins to different compartments of mitochondria is known to occur through TOM/TIM complexes [13,14]. Similarly, many nuclear encoded RNAs are also known to be imported to mitochondria [15]. The translocation of tRNA to mitochondria is known for long time [16]. 5S rRNA is also translocated to mitochondria from cytosol with help of rhodanese and L18; assembles into mitochondrial ribosomes [17]. Nuclear encoded ncRNA components of RNase P and MRP (mitochondrial RNA processing) enzyme complexes are translocated to mitochondrial matrix to cleave polycistronic mRNA and initiate mitochondrial DNA replication respectively [18,19]. The PNPASE (polynucleotide phosphorylase) complex involved in translocation of these RNAs has also been characterized [20]. These evidences suggest that there is an active transport of nuclear encoded RNAs to/in mitochondria.

Emerging evidences also suggest that the mitochondrial membrane serves as a novel platform to assemble RNA granule comprising of the proteins and RNA involved in RNA metabolism [21]. Mitochondria are also known to have dynamic interactions with P bodies [22,23], nuage bodies [24] and stress granules [25].

These evidences suggest that mitochondrial membrane may anchor RNA binding proteins, which may bind to small RNAs (sRNAs) including miRNA. Recently, miRNAs have also been found in mitochondria isolated from different tissues and different organisms: 15 miRNAs in rat liver mitochondria [26], 20 miRNAs in mouse liver mitochondria [27], 20 miRNAs in mitochondria from human myotubes [28] and 13 miRNAs in mitochondria from HeLa [29]. However, the physical association of small non-coding RNA including miRNA with mitochondria has not been well studied. We planned to use deep sequencing method to systematically analyze the association of sRNA with mitochondria. The deep sequencing method of fractionated RNA has become one of the most powerful tools to discover novel sRNA [30]. This approach has been widely used in finding novel sRNA although it does not provide detailed information about their spatial or temporal regulation. Therefore, we planned to combine subcellular fractionation and deep sequencing method to identify sRNA specifically associated with mitochondria. The method revealed that sRNAs including miRNA, piRNA, tRNA, rRNA and repeat associated RNA are associated with mitochondria. We specifically focused on analysis of miRNA and found that mitochondria are one of the post-transcriptional destinations of miRNA.

Results

Isolation and Analysis of Mitochondrial RNA

To analyze the sRNA families associated with mitochondria, a highly purified fraction of mitochondria was prepared from HeLa and HEK293 cell lines. The purity of mitochondrial fraction was confirmed by western blotting (Figure 1A). It was observed that NDUFS2, a subunit of mitochondrial complex-I, is highly enriched in purified mitochondrial fraction whereas RPS9 was only detectable in total cell and not in purified mitochondrial fraction. Ribosomal protein S9 (RPS9) is S4P family protein, a component of 40S ribosomal subunit, which is localized mainly in nucleolus and cytoplasmic ribonucleic component [31] but not in mitochondria. Hence, RPS9 was used to determine nuclear and cytosolic contamination. This evidence strongly suggests that purified mitochondrial fraction was free from nuclear as well as polysomal contamination.

RNA was isolated from total cells and purified mitochondria from both HEK293 and HeLa. The quality of RNA was determined by RNA integrity number (RIN values). The total cellular RNA showed clear peak of 28S and 18S RNA at 50 sec and 43 sec with RIN values of 8.5 and 10 for HEK293 and HeLa respectively. The microfluidic electrophoresis of mitochondrial RNA showed several peaks. It suggests presence of sRNAs other than 28S and 18S therefore traditional RIN may not be valid for mitochondrial RNA integrity. The purity of mitochondrial RNA was also analyzed at RNA level by RT-PCR of β-actin, nuclear encoded gene and ND4, mitochondrial encoded gene. The nuclear encoded mRNA, β-actin was only detected in the RNA from total cells and was absent in the mitochondrial fraction (Figure 1B) whereas ND4 was detected both in mitochondrial as well as total cellular RNA (HEK293 and HeLa) as expected. This suggests that there is no non specific association of nuclear encoded RNA with mitochondria. The mitochondrial RNA purity was further analyzed by qPCR for cytosolic RNA contamination by analyzing the presence of two nuclear encoded mRNA (TRIM4 and MITA) and two mitochondrial DNA encoded (ND4 and CYB) mRNA were taken as positive controls. The mitochondrial encoded mRNA was enriched significantly in mitochondrial fraction as compared to cytosolic mRNA (Figure 1C). These two experiments also

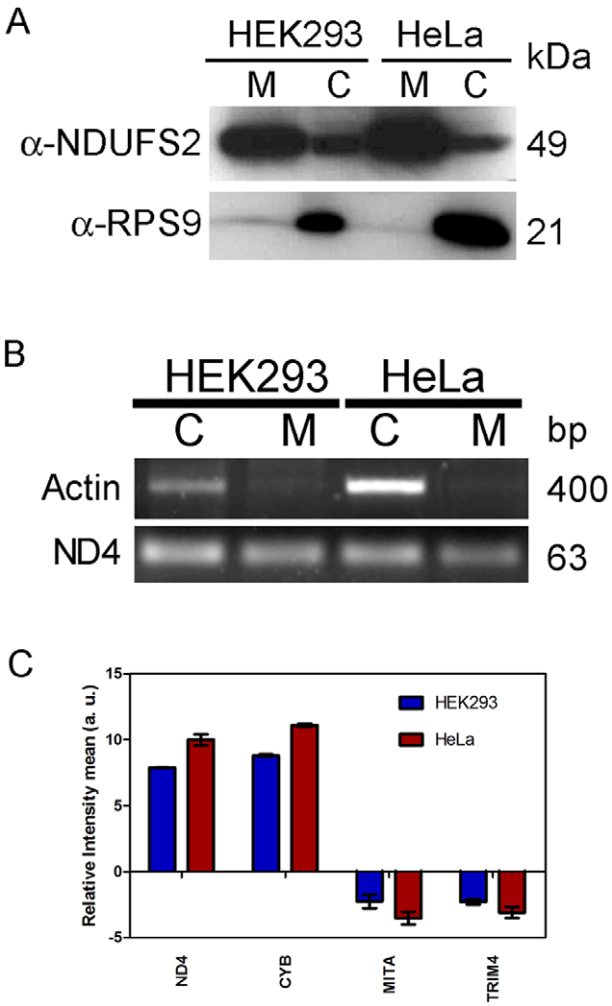


Figure 1. Analysis of isolated mitochondria. The mitochondria were isolated and purified from HEK293 and HeLa. (A) The protein contents of whole cell lysate, and purified mitochondria were normalized, resolved on 12.5% SDS-PAGE, transferred to PVDF membrane and probed with NDUFS2 and RPS9 antibody. (B) RNA was isolated from purified mitochondria and total cell. The subsequent cDNA was used for PCR amplification of mitochondrial encoded ND4 and cytosolic/nuclear specific β-actin. M: mitochondrial fraction; C: cellular lysate. (C) RNA was isolated from mitochondria. The nuclear RNA contamination in mitochondrial RNA was assessed by checking relative enrichment of mitochondrial encoded RNA (ND4, CYB) and nuclear encoded mRNA (TRIM4, MITA) by RT-qPCR as described in method section.
doi:10.1371/journal.pone.0044873.g001

suggest that mitochondrial RNA further analyzed by deep sequencing, is free from nonspecific association of nuclear encoded RNA.

Analysis of sRNA Libraries Associated with Human Mitochondria

The sRNA (18–30 nucleotides (nt)) associated with mitochondria was isolated, library generated and sequenced using Illumina high-throughput sequencing platform. The sequencing generated 19580503 and 17743919 raw sequencing reads associated with mitochondria of HEK293 and HeLa respectively. The dataset was deposited into NCBI Gene Expression Omnibus [accession No: GSM797669 and GSM797670 for sRNA associated with mito-

chondria of HEK293 and HeLa respectively]. Screening for 3′ adaptor sequences and 5′ adaptor contaminations using a local blastn program resulted in the elimination of 1749595 and 66668 tags from HEK293 and HeLa respectively. The reads of mRNA degraded products with poly-A sequence were low (350 and 215 reads each in HEK293 and HeLa respectively) indicating that the libraries were devoid of degraded mRNA products. The length of 731292 and 471296 reads in HEK293 and HeLa respectively were smaller than 18 nt, hence removed leaving behind 19089819 and 17312962 sequences for annotation of HEK293 and HeLa respectively.

The further analysis of sRNA library showed that 95% of total sequences (31686782 reads) corresponding to 10.52% unique sequence (104064 reads) were common in both HEK293 and HeLa. 3.87% total sequences (1290120 reads) corresponding to 63.3% unique sequences (626229 reads) and 1.04% total sequences (347483 reads) corresponding to 26.18% unique sequences (258992 reads) were specifically associated with mitochondria of HEK293 and HeLa respectively (Figure 2A, Figure 2B).

The length of miRNA, piRNA and siRNA are generally 21–22, 30 and 24 nt respectively [32]. Thus, the analysis of sRNA length helps to categorize the sRNA population obtained from both the cell lines. The length of 85.3% (14349816) and 91.2% (15070360) sequences from HEK293 and HeLa respectively were between 20–27 nt. The highest number of sequences were of 25 nt in length (17% and 27% sequences from HEK293 and HeLa respectively) (Figure 2C) indicating the abundance of sRNA other than miRNA and piRNA.

The sRNA sequences were mapped to human reference genome (UCSC hg19) to determine their origin and distribution. 87.58% (14728844 reads) and 92.67% (15297190 reads) from HEK293 and HeLa respectively aligned to the human genome in sense/antisense orientation. Majority of the sRNA reads aligned to the sense strand of uncharacterized region of genome (chrUn_g1000220) followed by antisense stands of chromosome 8 and 2 (Figure S1A, Figure S1B).

The sRNA sequences were annotated according to their overlap with sequences of known RNA in Genbank and Rfam (Figure 3A, Figure 3B, Table S1, Table S2). As expected, the most abundant sRNA classes in the both the libraries were rRNA (78.11% in HEK293 and 91.76% in HeLa), tRNA (3.96% in HEK293 and in 0.88% in HeLa) and unannotated sRNA (8.47% in HEK293 and in 3.27% in HeLa). The sequences corresponding to exons/introns were low (1.21% in HEK293 and in 0.11% in HeLa) suggesting the libraries were devoid of degraded mRNA products. The other categories of sRNA included snRNA (1.21% in HEK293 and 0.11% in HeLa), piRNA (0.01% in HEK293 and HeLa), srpRNA (0.17% in HEK293 and 0.01% in HeLa), snoRNA (0.21% and 0.03% in HEK293 and HeLa respectively) and repeat associated sRNAs. The top five abundant classes of repeats observed from mitochondria of both cell lines were rRNA (47.32% and 99.26% HEK293 and HeLa respectively), tRNA (1.63% and 0.36% HEK293 and HeLa respectively), LINES (0.104%and 0.057% HEK293 and HeLa respectively), SINE/Alu (0.15% and 0.028% HEK293 and HeLa respectively) and snRNA (0.32% and 0.072% HEK293 and HeLa respectively). Low levels of LTR/ERVL (0.015% and 0.007% HEK293 and HeLa respectively), LTR/ERV1 (0.015% and 0.007% HEK293 and HeLa respectively) and LTR/ERVL-MaLR (0.015%1 and 0.006% HEK293 and HeLa respectively) were also observed (Figure 3C, Figure 3D, Figure S2A, Figure S2B).

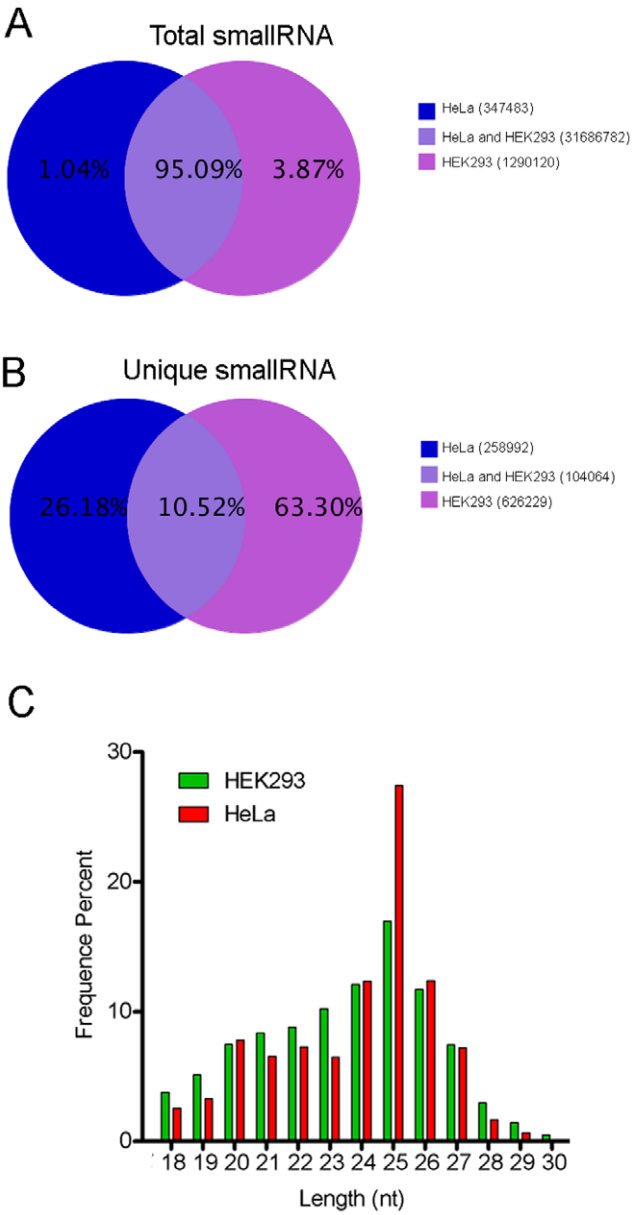


Figure 2. Generation and analysis of sRNA sequences from mitochondria. sRNA library generated from mitochondria from HEK293 and HeLa were sequenced using Illumina Hiseq 2000 platform that generated 19089819 and 17312962 clean sequence respectively. (A) Venn diagram showing distribution of common and specific sRNA total sequence reads amongst the two libraries. (B) Venn diagram showing distribution of common and specific sRNA unique sequence reads amongst the two libraries. (C) Length distribution and frequency percent of sequences in HEK293 and HeLa mitochondrial sRNA libraries.
doi:10.1371/journal.pone.0044873.g002

Analysis of known miRNAs Associated with Mitochondria

Our major interest was to investigate the association of miRNA with mitochondria hence we focused our analysis on miRNA. A total of 2249 unique tags (4.21% or 710742 sequence reads) and 1584 unique tags (2.58% or 426907 sequence reads) associated with mitochondria of HEK293 and HeLa respectively were categorized as miRNA (Table 1). The counts of miRNA varied from 1 to more than 100000. 428 and 327 mature miRNAs from HEK293 and HeLa respectively were observed to be associated

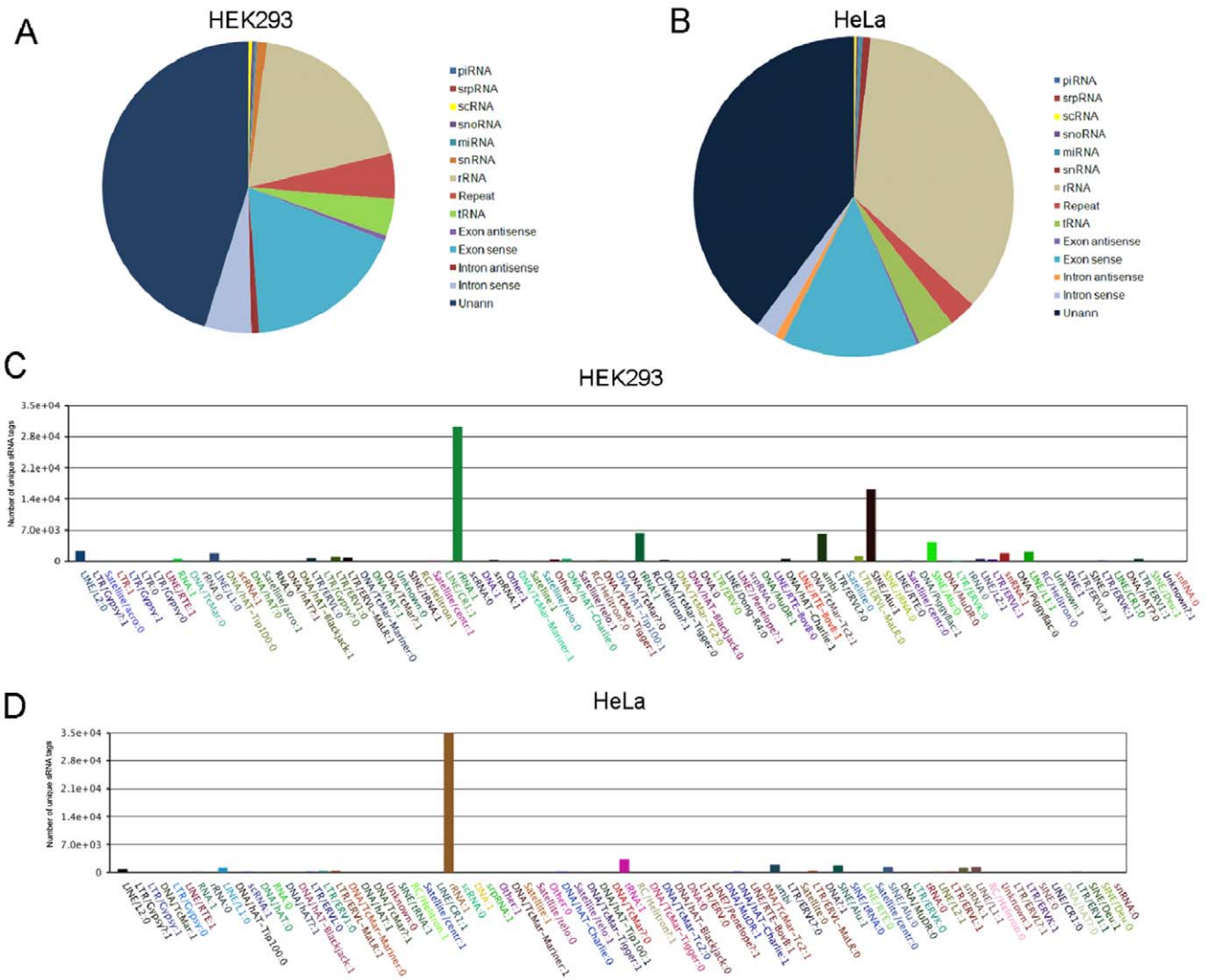


Figure 3. Frequency of distribution of different classes of RNA associated with mitochondrial sRNA libraries. The unique sequences obtained from the sRNA libraries were subjected to a series of sequence similarity searches using specific databases (rRNAs, tRNAs, snRNAs, snoRNAs, miRNAs, other non-coding RNAs). The sequences that did not match with any known sequence were considered as unannotated sequences. (A) An overview of sRNA associated with mitochondria of HEK293. (B) An overview of sRNA associated with the mitochondria of HeLa. The unique clean tags of repeat associated sequences were further categorized to determine the diversity of repeat associated RNA. (C) Detailed clustering of repeat-associated RNAs from mitochondria of HEK293. (D) Detailed clustering of repeat-associated RNAs from mitochondria of HeLa.
doi:10.1371/journal.pone.0044873.g003

with mitochondria. According to miRBase 17.0, we also found the association of 65 and 60 miRNA* with mitochondria of HEK293 and HeLa respectively.

Analysis of Differential Association of miRNAs with Mitochondria of HEK293 and HeLa

The differential association of miRNA to mitochondria of both HEK293 and HeLa has been summarized in Table S3. The

Table 1. Summary of known miRNAs from mitochondrial sRNA libraries.

	miRNA	miRNA*	unique sRNA matched to miRNA[1]	total sRNA matched to miRNA[2]
Known miRNA in miRBase	1539	194	–	–
HEK293	428	65	2249	710742
HeLa	327	60	1584	426907

The clean sequence reads aligned to miRBase 17.0 to determine diversity and occurrence of miRNA in sRNA library.
[1]count of unique sRNA sequences that matched to miRBase,
[2]count of total sRNA sequences that matched to miRBase in HEK293 and HeLa.
doi:10.1371/journal.pone.0044873.t001

frequency counts of 35 miRNAs associated with mitochondria of both the cell lines was similar (Figure 4A). The frequency count of miRNA ranged from 1 to more than 100,000 (Figure 4B). The most abundant miRNAs associated with mitochondria of HEK293 and HeLa were hsa-miR-423-5p, hsa-miR-320a and let-7 family members (let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7h and let-7i) followed by hsa-miR-103b, hsa-miR-140-3p, hsa-miR-744, hsa-miR-107 (Figure 4C, Figure 4D, Table S3). hsa-miR-10a, hsa-miR-128, hsa-miR-1307, hsa-miR-140-3p, hsa-miR-185, hsa-miR-196a, hsa-miR-25, hsa-miR-320a, hsa-miR-330-3p, hsa-miR-340, hsa-miR-423-5p, hsa-miR-629 and hsa-miR-744 significantly associated with the mitochondria of HEK293. Similarly let-7i, hsa-miR-181b, hsa-miR-21, hsa-miR-23a, hsa-miR-29a, hsa-miR-30a, hsa-miR-31 and hsa-miR-452 associated significantly with mitochondria of HeLa.

Analysis of Putative Novel miRNAs Associated with Human Mitochondria

As described above we observed significant number of reads from unannotated region of chromosome. The unannotated sequences were analyzed through computational pipeline described in methods to classify them as putative novel miRNA. In total, 196 and 13 putative novel miRNAs were identified by virtue of their ability to form miRNA-like single hairpins and other criteria of miRNA from the mitochondrial sRNA libraries of HEK293 and HeLa respectively (Table S4). Here, we used the naming convention "293m8-miRX" and "HM3-miRX" for each putative novel miRNAs associated with mitochondria of HEK293 and HeLa respectively. The counts of putative novel miRNAs ranged from 5–3132 in HEK293 and 5–208 in HeLa (Figure 5A). Interestingly, some putative novel miRNAs had similar sequence, count and structure but mapped to different genomic locations. Analysis of the first nucleotide bias of the 18–30 nt miRNA candidates revealed that uridine (U) was the most common base (>90%) at positions 1, 19, 20, 21, 22 of 22 nt long putative novel miRNAs (data not shown). The analysis of 20–24 nt miRNAs showed that the first nucleotide of 40% of 20 nt long miRNAs started with G, 40% of 21 nt long miRNAs with U, >90% of 22nt long miRNAs with U, >50% of 23 nt long miRNAs with A and >60% of 24 nt long miRNAs with U in HEK293 (Figure 5B). Interestingly, in HeLa 100% of 20 nt long miRNAs began with A, 60% of 21 nt long miRNAs with A, 40% of 21 nt long miRNAs with U, >95% of 22 long miRNAs with U and 100% of 23 nt long miRNAs with G (Figure 5C).

Alignment of sRNAs to Human Mitochondrial Genome

Human mitochondria contain 16.5 kb circular DNA as its own genome. We further studied the alignment of sRNAs with mitochondrial genome. We used two different approaches to search for the sequences that aligned to mitochondrial genome.

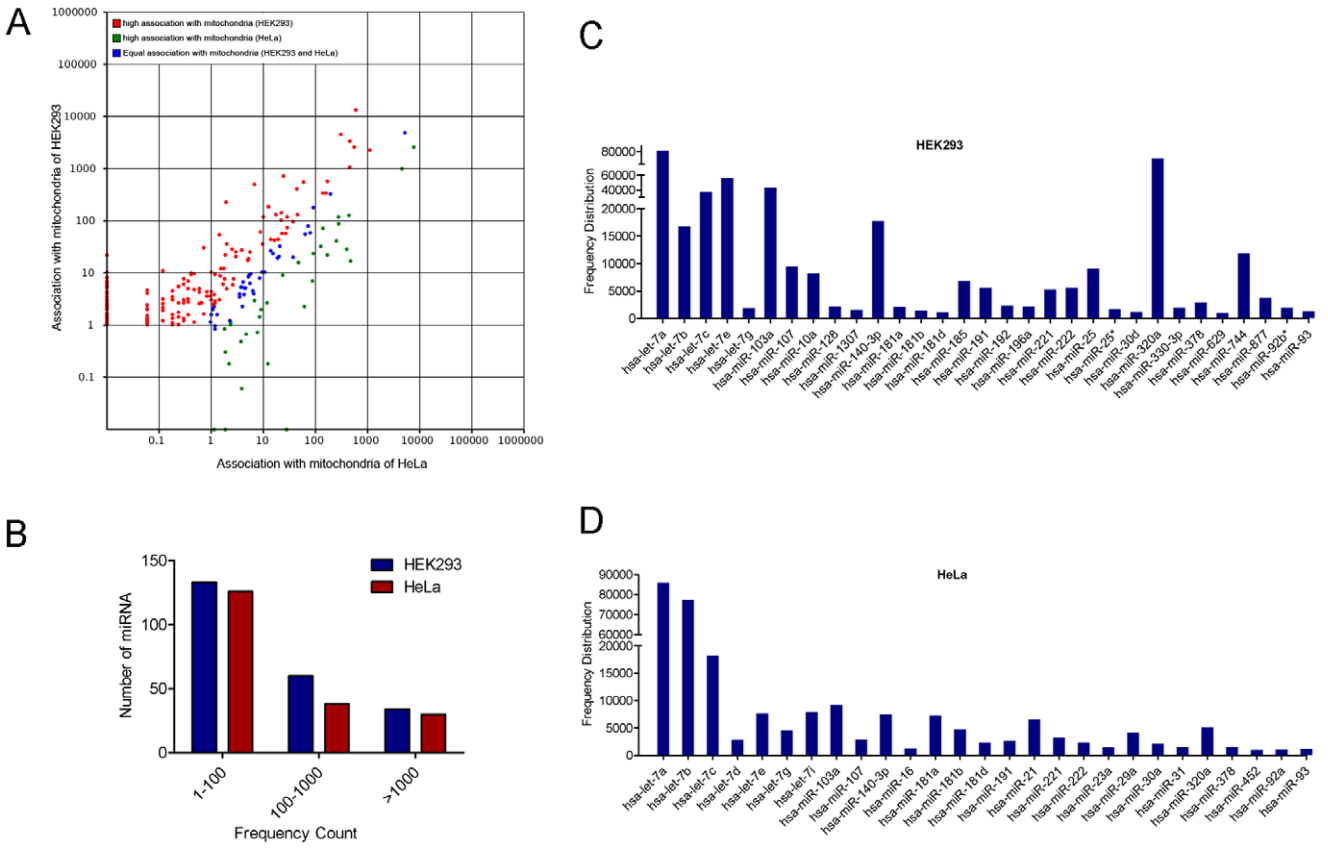


Figure 4. Analysis of differential association of miRNAs to mitochondria from HEK293 and HeLa. (A) Scatter Plot depicting the differential association of miRNAs from libraries of HeLa and HEK293. The X and Y axis shows association level of miRNAs with mitochondria from two cell lines. Red points represent miRNA with ratio>2; Blue points represent miRNA with 1/2<ratio<2; Green points represent miRNA with ratio<1/2. Ratio = normalized association of the HEK293/HeLa. (B) Distribution of known miRNAs: Numbers of sequence reads are taken as miRNA levels and the values are represented in the form of range of values in both HEK293 and HeLa sRNA libraries. (C) The frequency of highly associated miRNAs (>10,000 counts) with mitochondria of HEK293. (D) The frequency of highly associated miRNAs (>10,000 counts) with mitochondria of HeLa.
doi:10.1371/journal.pone.0044873.g004

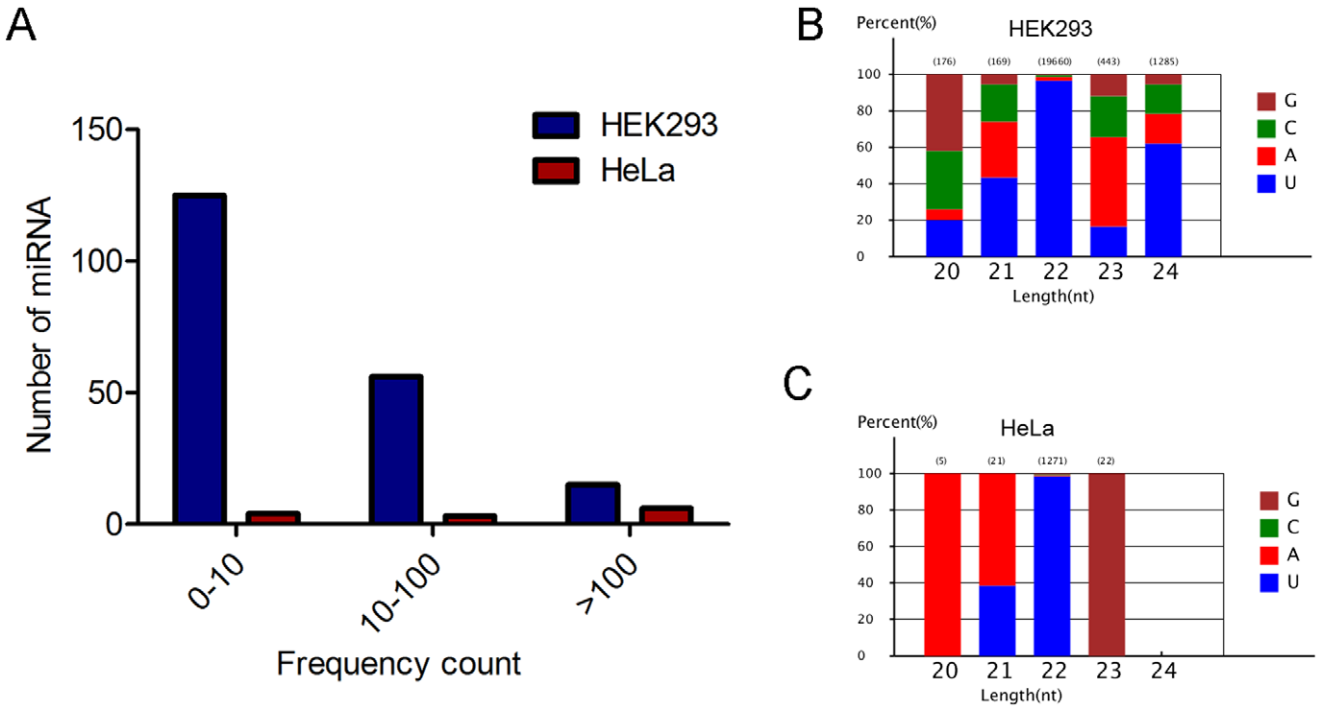


Figure 5. Analysis of putative novel miRNAs associated with human mitochondria. The putative novel miRNAs were predicted from unannotated clean reads using Mireap software. (A) The distribution of putative novel miRNAs levels with respect to frequency. Numbers of sequence reads are taken as miRNA levels and the values are represented in the form of range of values in both HEK293 and HeLa mitochondrial sRNA libraries. (B) The percentage of first nucleotide base bias of 18–23 nt putative novel miRNAs from mitochondria-associated sRNA library of HEK293. (C) The percentage of first nucleotide base bias of 18–23 nt putative novel miRNAs from mitochondria-associated sRNA library of HeLa.
doi:10.1371/journal.pone.0044873.g005

Firstly, SOAP parameters described in method section, showed that 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 aligned sequences belonged to unannotated (40.9% in HEK293 and 16.8% in HeLa), tRNA (38.2% in HEK293 and 40.1% in HeLa) and rRNA (16.4% in HEK293 and 39.4% in HeLa) categories. Some sequences were also categorized as repeat associated sequences (0.36% in HEK293 and 0.14% in HeLa) and miRNA (0.01% in HEK293 and 0.035% in HeLa) (Figure 6A, Figure 6B). The analysis showed that only four miRNAs: 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) corresponding to ND4L, ND5, L-ORF and 16S rRNA genes respectively. Analysis using another tool MapMi version 1.0.4.02 [33] resulted in alignment of 744 unique sequences to mitochondrial DNA. We also used local BLASTN version 2.2.20 program to screen putative novel miRNA alignments. We found the alignment of first 11 bases of 24 putative novel miRNAs at different positions of mitochondrial genome (Figure 6C). The 7 putative novel miRNAs aligned to non coding region, 5 to ATP6, 3 to tRNA, 2 to 12S rRNA, 2 to ND2 and 1 to HVRI, COI, CytB and ND1 gene region.

Biological Processes Regulated by miRNAs Associated with Mitochondria

An individual miRNA may regulate several mRNAs in a pathway thus fine-tuning the cellular processes. Majority of target prediction tools give many false positive results therefore it has to be validated by both computational and experimental tools. We used Starbase that has been developed based on recent studies of CLIP (Cross-linking immunoprecipitation) and RNA degradome sequencing experiments (high-throughput sequencing data from 21 CLIP-Seq and 10 Degradome-Seq experiments from six organisms) [34]. The false positives targets were further eliminated by taking intersections of targets from three prediction tools. Therefore, the outcome obtained from this analysis may result in reliable targets mimicking closely to the physiological conditions.

Identified miRNAs were divided in two subgroups on the basis of frequency of association with mitochondria from HEK293 and HeLa: highly abundant miRNAs (count >5000) and less abundant miRNAs (count <5000). The targets of these miRNAs were further analyzed using DAVID to functionally annotate the identified targets into smaller and biologically meaningful groups. The targets of highly abundant miRNAs populated many GO categories with significant enrichment (>1.0) and p-Value (<0.05). GO terms related to regulation of transcription (GO: 0045449, GO: 0010629) were significantly enriched. The other significantly enriched categories included GO: 0051301~cell division, GO: 0007049~cell cycle, GO: 0016568~chromatin modification, GO: 0035195~gene silencing by miRNA, GO: 0007389~pattern specification process, GO: 0001701~in utero embryonic development, GO: 0042921~glucocorticoid receptor signaling pathway (Table S5). To analyze the role that miRNAs play in the regulatory networks, we assigned putative miRNA targets into KEGG pathways, and observed that ubiquitin mediated proteolysis pathway was significantly enriched (p-value <0.05) (Table S6).

The GO analysis of the targets of less abundant miRNAs associated with mitochondria (<5000) showed significant enrichment of GO terms related to regulation of transcription (GO: 0045449, GO: 0010629, GO: 0006357, GO: 0051252) and the regulation of biosynthetic processes (GO: 0031327/28, GO:

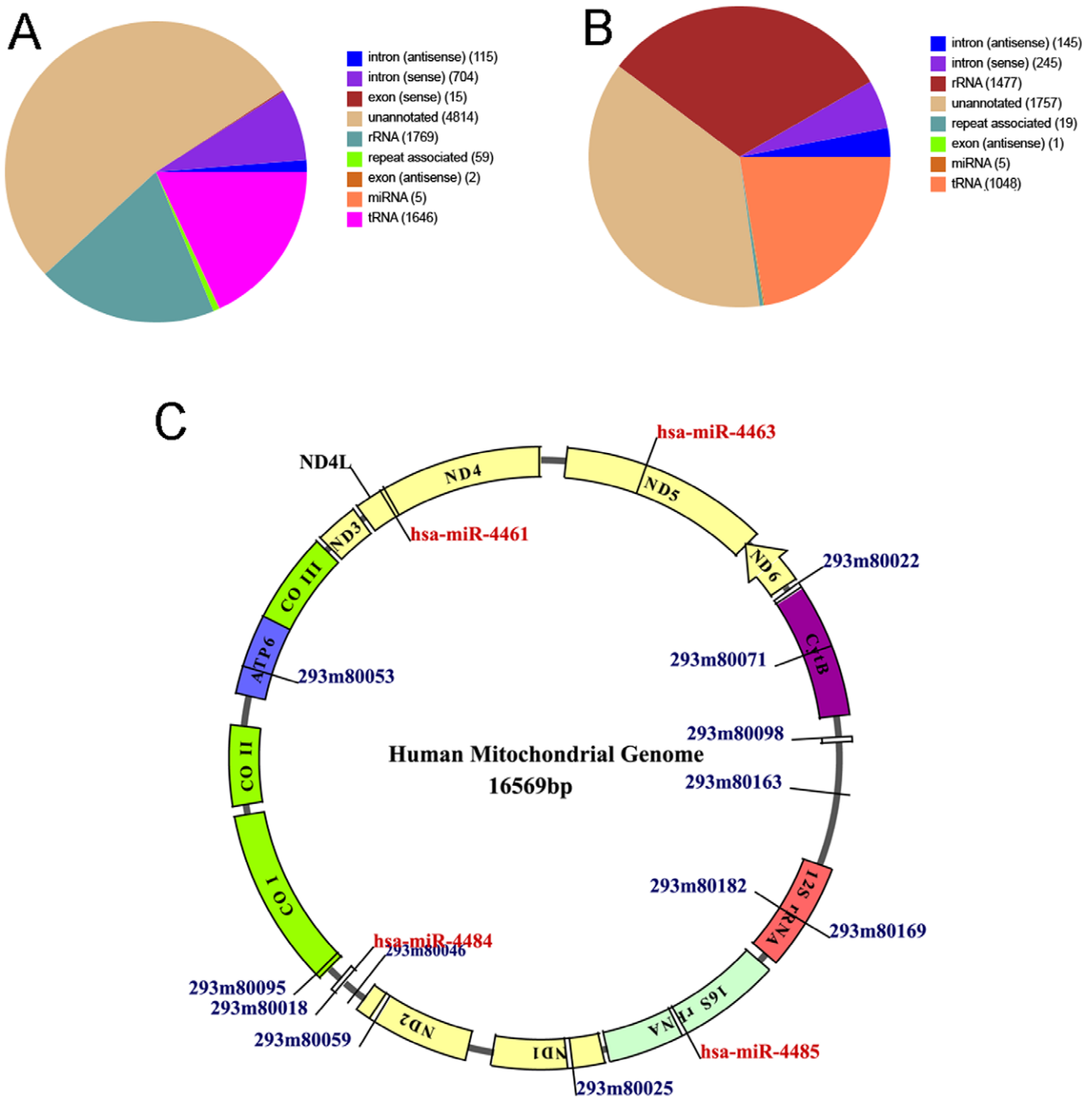


Figure 6. Mapping sRNAs to human mitochondrial genome. The sequences obtained from the mitochondrial sRNA libraries from HEK293 and HeLa were aligned to mitochondrial genome. (A) An overview of mitochondria-associated sRNAs from HEK293 that aligned to mitochondrial genome. (B) An overview of mitochondria-associated 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.
doi:10.1371/journal.pone.0044873.g006

0009891) (Table S7). To further analyze the networks we also used KEGG and found that pathways in cancer (solid tumors) and chronic myeloid leukemia were significantly enriched ($p<0.05$). Similarly TGF, Wnt and cell cycle pathway genes were also found be to significantly enriched ($p<0.05$) (Table S8).

The targets of putative novel miRNAs associated with mitochondria of HEK293 showed enrichment for positive/negative regulation of transcription (GO: 0006350~transcription, GO: 0051252~ RNA metabolic process, GO: 0010629~negative regulation of gene expression). The nucleic acid, protein metabolic and catabolic related GO terms were also found to be enriched (GO: 0051254~positive regulation of RNA metabolic process, GO: 0045935~positive regulation of nucleic acid metabolism, GO: 0030163~protein catabolic process, GO: 0019220~regulation of phosphate metabolic process, GO: 0032268~regulation of cellular protein metabolic process) (Table S9). The KEGG analysis

of the identified targets of putative novel miRNAs associated with mitochondria of HEK293 showed the regulation of neurotrophin signaling, cell cycle, phosphatidyl inositol signaling system (Table S10).

Similarly, targets of putative novel miRNAs associated with mitochondria from HeLa cells were also analyzed. The GO term related to apoptosis, cell cycle, stress response (GO: 0042981~regulation of apoptosis, GO: 0051726~regulation of cell cycle cellular processes, GO: 0000079~regulation of cyclin-dependent protein kinase activity, GO: 0001938~positive regulation of endothelial cell proliferation) were the enriched clusters (Table S11). KEGG analysis of identified targets of putative novel miRNAs associated with mitochondria from HeLa showed the gene network involved in endocytosis, p53 signaling, adherence junction, dilated cardiomyopathy and cancer (Table S12).

Validation of known miRNAs and Target mRNAs Associated with Human Mitochondria

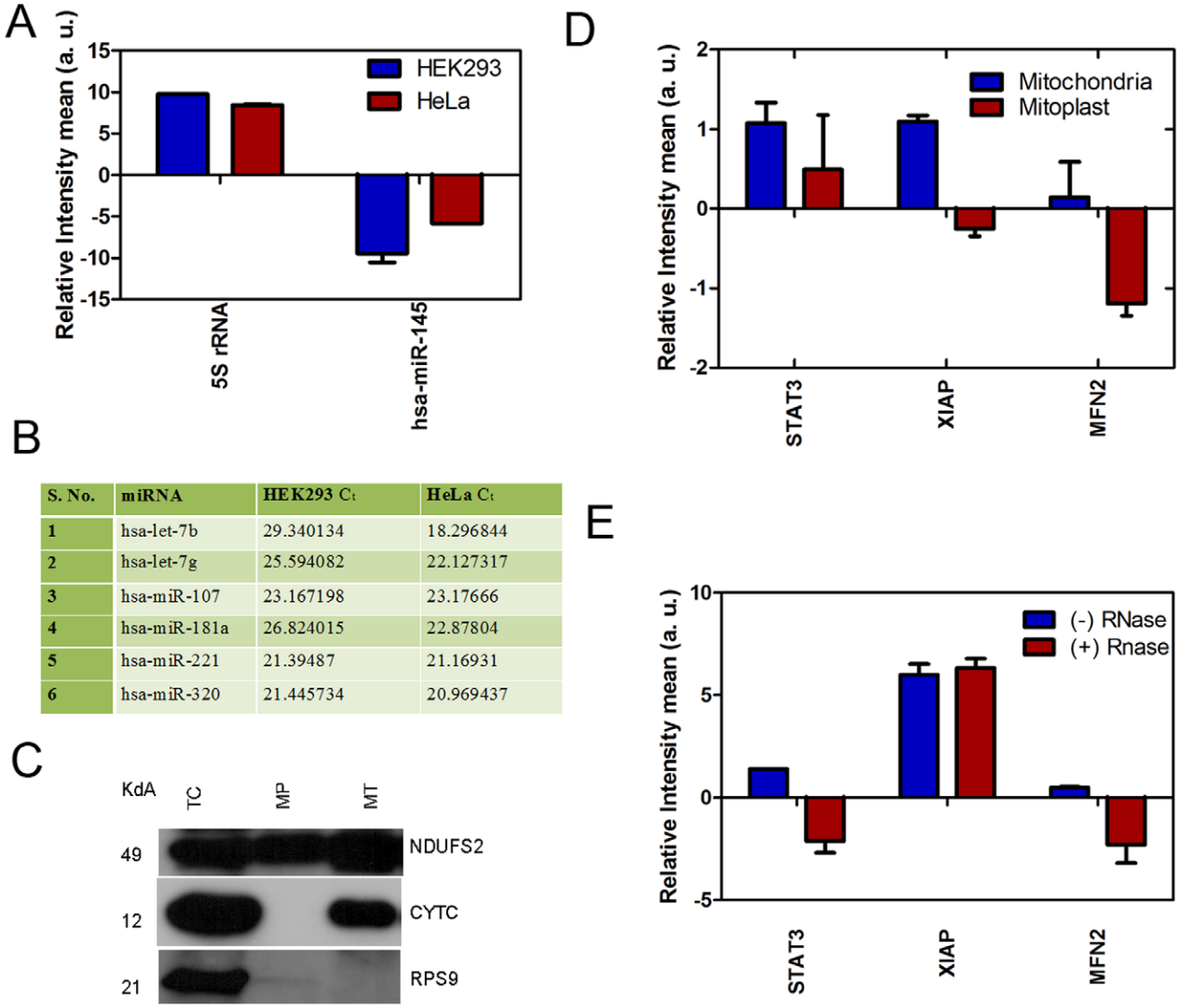
The experiments were done to validate the association of miRNAs with mitochondria. The non specific association of miRNA was excluded by analyzing controls based on previous experiments. U6 snRNA and 5S rRNA was taken as endogenous and positive controls respectively due to their known association with mitochondria [29], [27]. We also observed high levels of U6 in our sRNA microarray analysis of mitochondrial RNA from HEK293 (data not published). Our microarray results also demonstrated that hsa-miR-145 was not detected in mitochondrial fraction whereas predominantly present in the total cell suggesting that it is not associated with mitochondria, hence was taken as negative control. The analysis by real time PCR showed that 5S rRNA was significantly associated whereas no association of hsa-miR-145 was observed in the RNA fraction isolated from purified mitochondria (Figure 7A). This experiment here as well as results from previous experiment clearly showed that specific miRNA associate with mitochondria and there is no non-specific association. The miRNAs with high count from both libraries (let-7b, let-7g, hsa-miR-107, hsa-miR-181a, hsa-miR-221 and hsa-miR-320a) were considered for the analysis for their association with mitochondria. The miRNAs assessed by RT-qPCR followed similar pattern of association (Figure 7B) supporting the sequencing results. Argonaute proteins bind to guide strand of mature miRNA and regulate the process of translation. The association of miRNA to mitochondria also suggests that Ago proteins may also be localized to mitochondria hence we analyzed the subcellular localization of Ago2/3 proteins. We observed that Ago2 colocalized with mitochondria (Figure S3) which was in consonance with earlier observations [27,29]. Ago3 also colocalized with mitochondria whereas GFP vector (negative control) showed no co-localization with mitochondria.

We hypothesized that if these miRNAs and miRISC components are associated with mitochondria, the target mRNAs may also be associated to the outer surface of mitochondria. We analyzed the targets of 3 miRNAs (let-7b: STAT3; hsa-miR-107: MFN2; hsa-miR-320a: XIAP) by StarBase using intersection of 3 computational tools as described in materials and methods and checked their association with mitochondria. To check the association of miRNA/target mRNA with outer membrane the levels of target mRNAs were analyzed by qPCR in both mitochondria and mitoplast. The mitoplast preparation was checked prior to isolation of RNA by western blotting. The inner membrane localized protein, NDUFS2 was significantly enriched in mitoplast whereas, the inter membrane space protein cytochrome c was absent in mitoplast (Figure 7C). The levels of target mRNAs (STAT3, MFN2 and XIAP) of selected miRNAs (let-7b, hsa-miR-107, hsa-miR-320a) were significantly associated with mitochondria whereas the levels decreased significantly in mitoplast (Figure 7D). This was also confirmed by analyzing the association of mRNAs with mitochondria after RNase A treatment. RNase A treatment resulted in significant decrease in levels of target mRNAs (STAT3 and MFN2) whereas the levels of XIAP remained unchanged (Figure 7E). This may be due to association of XIAP mRNA with RNP complexes or localization site in mitochondria which may be not be accessible by RNase A for degradation. These evidences suggest that the subunits of RISC complex like Ago proteins localize to the outer membrane may bind to target mRNA and may serve as platform for assembly of site-specific regulation of mRNA turnover and protein levels.

Discussion

The import of nuclear encoded tRNA and rRNA into the mitochondria has been demonstrated [17,35]. This raised an interesting question of possible import of small non-coding RNAs including miRNA to the mitochondria, which may fine-tune the levels of target proteins associated with mitochondria and under various physiological conditions. In this study, we described the association of sRNAs including miRNA with mitochondria of two human cell lines HEK293 and HeLa through deep sequencing. The targets of identified known and putative novel miRNAs may regulate critical cellular pathways where mitochondria plays key role.

The deep sequencing of mitochondrial associated RNA confirmed the presence of diverse population of sRNAs: snRNA, srpRNA and snoRNA. The association of diverse population of sRNAs is intriguing and suggests either the role of mitochondria in their biogenesis or the role of sRNA in regulation of mitochondrial functions. The snRNA and snoRNA are transported back from cytoplasm to sub-nuclear regions [36]. It has been observed that snoRNA are processed in a similar manner like miRNA [36] and may perform functions similar to miRNA apart from its role in ribosomal biogenesis. The association of snoRNA with mitochondria needs to be further studied for their role in mitochondrial ribosome assembly, RNA modifications or miRNA like functions. The association of srpRNA also suggests that mitochondria may be involved in the translational of the mRNA encoding mitochondrial targeted proteins. These observations were further strengthened from the recent study describing the mitochondrial transcriptome where snRNA, snoRNA and srpRNA were observed to be associated with mitochondria [37]. Interestingly, we also observed piRNA associated with mitochondria from both HEK293 and HeLa cells. It has been observed earlier that piRNAs are generally expressed in germ line tissues but emerging evidences suggest the presence of piRNA in different somatic tissue [38]. The expression of piRNA was observed in 17 out of the 40 mouse somatic tissues and cell types. The piRNA expressed in somatic tissues showed characteristics of piRNA such as length distribution between 24 and 32 nt, 5′ uridine bias and clustering along the genome along with universal piRNA features. The association of piRNA with mitochondria of HEK293 and HeLa in this study suggests either mitochondria are involved in biogenesis of piRNA or postranscriptionally associates with mitochondria like miRNA. The role of mitochondria in biogenesis of piRNA has been further supported by recent studies. Two groups have clearly demonstrated the role of mitochondrial protein known as MitoPLD/zuc (Drosophila homologue) in piRNA biogenesis and piRNA mediated silencing both in fly and mouse germlines [23,39]. This suggests that mitochondria may be an important player in piRNA pathways.



S. No.	miRNA	HEK293 Ct	HeLa Ct
1	hsa-let-7b	29.340134	18.296844
2	hsa-let-7g	25.594082	22.127317
3	hsa-miR-107	23.167198	23.17666
4	hsa-miR-181a	26.824015	22.87804
5	hsa-miR-221	21.39487	21.16931
6	hsa-miR-320	21.445734	20.969437

Figure 7. Validation of miRNAs and their targets associated with mitochondria. The enrichment of target mRNA of mitochondria-associated miRNAs with outer mitochondrial membrane were validated. (A) The association of 5S rRNA and negative control (hsa-miR-145) to mitochondria was analyzed. The relative enrichment of 5S rRNA and hsa-miR-145 was determined by qPCR as described in methods. (B) The miRNAs (frequency >1000) which were common in both the libraries were selected for validations by RT-qPCR. RNA was prepared from purified mitochondria and cDNA synthesized. The mean CT values of miRNAs (with CT <30 and distinct melt curve) from the mitochondria of both HEK293 and HeLa are listed. (C) The mitoplast was prepared from HEK293 as described in method section. The purity of mitoplast preparation was assessed by western blotting by probing with antibody against NDUFS2 (mitochondrial inner membrane protein) and cytochrome c (inter membrane space protein). TC: total cell; MP: mitoplast; MT: mitochondria. The targets of 3 miRNAs associated with mitochondria (let-7b: STAT3; hsa-miR-107: MFN2; hsa-miR-320a: XIAP) were determined by Starbase and validated by qPCR. (D) Validation of target mRNA associated with mitoplast as compared to mitochondria from HEK293. (E) Validation of target mRNA associated with RNase A treated mitochondria from HEK293.
doi:10.1371/journal.pone.0044873.g007

Interestingly, 2–5% sRNA reads was categorized to be miRNA supporting the recent evidence of existence of mitochondrial associated miRNA [26,28,29]. During our manuscript preparation, mitochondrial transcriptome was analyzed [37] which is in consonance with our study and observed that 3% of sRNAs showed similarity to miRNA. Interestingly, we observed that miRNA identified in recent studies [28,29,37] as well as microarray of mitochondrial RNA from our lab (data not published) was found to be widely represented in our deep sequencing results. This strongly suggests that our deep sequencing approach confirmed the validated results observed in the previous studies. The further analysis showed that the frequency of association of 35 miRNAs with mitochondria of HEK293 and HeLa were similar whereas majority of miRNAs were differentially associated in both cell lines. Similarly, 196 and 13 putative novel miRNAs were associated with mitochondria from HEK293 and HeLa respectively. These evidences suggest that association of miRNAs with mitochondria may be cell type and stimulus specific which needs further analysis using different cell lines and in different conditions. Interestingly, base biasness of miRNA (22 nt) showed preference for U at the first position in both HEK293 and HeLa suggesting mitochondrial associated miRNA may have high affinity to preferentially bind to MID domain of Ago2 and may be involved in binding with targets for gene regulation [40]. Further,

it was observed that 4 known and 24 putative novel miRNA aligned to mitochondrial genome at different positions corresponding to 16S rRNA, tRNA and subunits of complex I. These miRNAs may be either mitochondrial genome encoded or nuclear encoded, may regulate mitochondrial transcripts involved in ribosome assembly and electron transport chain. This will require further experimental validations.

We identified the targets of miRNAs associated with mitochondria with high frequency count from both HEK293 and HeLa. Interestingly, GO terms related to RNA transcription were enriched. Ago2, core component of RISC complex involved in regulation and turnover of target mRNA has been reported to be colocalized with mitochondria [41]. Our results also showed the association of Ago2 and Ago3 with mitochondria (Figure S3). It has been well established that Ago proteins associates with miRNA [42]. Interestingly, Ago1–3 has recently been demonstrated to be redundantly associated with diverse population of RNA apart from miRNA including tRNA, vRNA, snoRNA, rRNA pseudogenes, snRNA, tiRNA, TSSa-RNA, PASRs, etc [43] which may be one of the reasons explaining the observed association of these RNAs to mitochondria. These sRNAs are known to regulate mRNA turnover and translation which again emphasizes the possible role of mitochondria in these processes. Similarly, it has been observed that mitochondria also associate with P bodies [44]. These P bodies are cytoplasmic granules that are linked to mRNA decay, mRNA storage and RNA silencing. In our study, we also observed that miRNAs and target mRNAs associated with mitochondria were enriched to the outer membrane of mitochondria. This is also supported by recent study of mitochondrial transcriptome where depletion of outer membrane of mitochondria results in selective loss of miRNAs [37] and other RNAs. These evidences strongly suggest that the outer membrane may assemble novel complexes by associating the proteins of RISC complexes, target mRNA to regulate site-specific turnover of mRNA and translation.

Analysis of putative novel miRNAs associated with mitochondria from HEK293 and HeLa showed enrichment of GO functions related to positive/negative regulation of RNA which has been discussed above that further supported our hypothesis. Interestingly, putative novel miRNAs identified in our study also showed enrichment of GO functions related to several signaling pathways important in cancer and metabolism where mitochondria plays crucial role. Apoptosis, cell cycle, kinase activity and proteins transport related pathways were enriched. Some of the known miRNA-target interactions have been demonstrated previously. hsa-miR-103/107 is highly associated with mitochondria (as observed in our study) which regulates cellular Acetyl-CoA and lipid levels [45]. Similarly hsa-miR-181 targets multiple Bcl-2 family members and regulates apoptosis [46]. These evidences suggest that miRNAs associate with mitochondria and regulates the turnover of mRNA and levels of protein that are implicated in mitochondrial related functions.

In conclusion, our study using subcellular fractionation and deep sequencing demonstrated the association of sRNAs including miRNA to mitochondria. The putative novel miRNAs needs further characterization for their function, association with mitochondria and translocation to other subcellular sites during different cellular processes. The association of miRNAs, their target mRNAs and Ago2/3 with mitochondria suggests that mitochondrial outer membrane may probably provide novel platform to assemble the miRNA/RISC complexes to regulate the subcellular site-specific protein levels. The further study in this direction will help to understand many unanticipated role of mitochondria and associated miRNA in different physiological and pathological conditions.

Materials and Methods

Cell Culture

HEK293 and HeLa cells were grown at 5% CO2, 37°C in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Invitrogen, USA) supplemented with 1% penicillin, streptomycin and amphotericin B (PSA) antibiotic mixture (Gibco, Invitrogen, USA) and 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Invitrogen, USA). HEK293-MTRFP stable cell line was generated as described in Methods S1.

Preparation and Analysis of Purity of Mitochondria

Mitochondria were prepared and purified from HEK293 and HeLa cells using Qproteome Mitochondria Isolation Kit (Qaigen, USA) as per manufacturer's instructions with minor modifications. Briefly, 5×10^8 cells were suspended in Lysis buffer incubated in ice for 10 min and centrifuged at 1000× g for 10 min at 4°C. The pellet was washed again with Lysis buffer and resuspended in Disruption Buffer, passed 10 times through 24 gauge needle to ensure complete cell disruption and centrifuged at 1000×g. The supernatant was centrifuged at 8000 rpm for 10 min at 4°C, to pellet down mitochondria. The mitochondria were washed and purified by adding on top of layers of Purification Buffer and Disruption Buffer. The solution was centrifuged at 13000 rpm for 15 min at 4°C. The mitochondrial ring at the interface of Purification Buffer/Disruption Buffer was collected and washed in Mitochondria Storage Buffer.

The purity of the mitochondrial fraction was assessed by western blotting as described earlier [47] with minor modifications. Briefly, the mitochondria and total cells were solubilized in solubilizing buffer (50 mM Bis-Tris (pH 7.0), 750 mM e-aminocaproic acid, 0.1% TritonX-100 and 10 ug/ml protease inhibitor). The concentration of total cellular and mitochondrial lysate was determined by Bradford assay, normalized and resolved on 12.5% SDS-PAGE. The proteins were electroblotted on PVDF membrane at 100 V for one hour. The membrane was blocked with 5% non-fat dried milk and 0.1% Tween-20 in PBS for 1 hour at room temperature. The membrane was incubated with rabbit polyclonal antibody against NDUFS2 (mitochondrial complex I subunit). After incubation membrane was washed three times with PBS-T (PBS containing 0.1% Tween 20) for 10 minutes each and incubated with a secondary antibody at room temperature for 1 hr. The membrane was again washed three times with PBS-T and signal visualized by using EZ-ECL chemiluminescence detection kit (Biological Industries, Israel) by exposing to X-ray film. The membrane was restriped and further probed with polyclonal antibody against RPS9 (ribosomal protein S9). All primary antibodies were used at 1:1000 dilutions.

The nuclear contamination and mitochondrial enrichment were checked at RNA level by RT-PCR. RNA was isolated from mitochondria and first strand cDNA was synthesized using iScript^{TM}cDNA Synthesis Kit (Bio-Rad, USA) at 42°C for 1 hour, followed by inactivation of enzyme at 85°C for 5 min. The β-actin (Fwd: CTGGAACGGTGAAGGTGACA; Rev: AAGGGACTTCCTGTAACAATGCA) gene was taken as nuclear control whereas mitochondrial DNA encoded mRNA as positive control (ND4 (Fwd: ACAAGCTCCATCTGCCTACGA; Rev: GGCTGATTGAAGAGTATGCAATGA)) for PCR amplification. The reactions were performed at annealing temperature of 56°C.

RNA Isolation from Purified Mitochondria

RNA was extracted from purified mitochondria and total cell using miRCURY^{TM} RNA Isolation Kit according to manufac-

turer's instructions (Exiqon, Germany). Briefly, the purified mitochondria were resuspended in RNA lysis buffer and incubated at room temperature in column for 5 min to allow binding. The column was washed thrice with washing buffer at 14000 rpm for 1 min. The RNA was eluted in nuclease free water from the column. Purity and concentration of RNA was determined using NanoPhotometer (Implen, Germany). The integrity of mitochondrial and total cellular RNA was checked with Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., USA).

The cytosolic mRNA contamination was checked by RT-qPCR. The mitochondrial encoded mRNAs: ND4 (Fwd: ACAAGCTCCATCTGCCTACGA; Rev: GGCTGATTGAAGAGTATGCAATGA) and CYB (Fwd: AACCGCCTTTTCATCAATCG; Rev: AGCGGATGATTCAGCCATAATT); cytosolic mRNAs: TRIM4 (Fwd: CACCAGACTCACGCCATG; Rev: ACGAGATTACGCTGAGACTTAAG) and MITA (Fwd: CGCCTCATTGCCTACCAG; Rev: ACATCGTGGAGGTA CTGGG) were relatively quantified taking RNP (Fwd: CCCC GTTCTCTGGGAACTC; Rev: TGTATGAGACCACTCT TTCCCATA) as endogenous control using SYBR® Premix Ex Taq™ (Takara, Japan) as per manufacturer's instruction. Briefly, the initial denaturing was done at 95°C for 30 sec followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The specificity of each amplicon was checked by melt curve analysis. The reactions were performed using StepOnePlus™ (Applied Biosystem, USA). The reactions were performed in triplicates, $\Delta\Delta C_T$ calculated and relative association with standard error was plotted using Graph Pad PRISM 5.0.

Isolation of Mitoplast

The mitoplast was prepared from purified mitochondria as described earlier [48] with minor modifications. The mitochondria (protein concentration of 1.0 mg/ml) were incubated in 10 mM KH_2PO_4 with 2.7 mg/ml digitonin for 20 min on ice. The solution was centrifuged at 10,000 g for 10 min at 4°C. The pellet was washed twice with PBS. The quality of preparation was confirmed by analyzing the presence/absence of cytochrome c by western blotting as described earlier.

RNase A Treatment

The mitochondria were treated with RNase A to check the association of miRNAs and mRNA with outer surface of mitochondria as described previously [28]. The purified mitochondrial pellet was resuspended in 20 µl mitochondrial storage buffer and mixed with 300 µl buffer P1 (QIAprep Spin Miniprep Kit, Qiagen, USA) containing 40 µg/ml RNase A and incubated at 37°C for 1 hr. The reaction was stopped by adding Proteinase K (100 mg/ml) resuspended in PBS. RNase A treated mitochondria were pelleted down at 8000 rpm for 10 min and washed twice in mitochondrial storage buffer prior to RNA isolation.

sRNA Library Preparation and Sequencing

RNA library preparation and sequencing was performed by BGI (Beijing Genomics Institute, Shenzhen, China). Briefly, sRNA (18 to 30 nt) was gel purified and ligated to the 3′ and 5′ adaptor. The ligated products were used for cDNA synthesis, followed by acrylamide gel purification and PCR amplification for 14 cycles of 98°C for 10 sec and 72°C for 15 sec to generate sRNA libraries [30]. The library (1 µl) was loaded on an Agilent Technologies 2100 Bioanalyzer to check size, purity, and concentration. Libraries were sequenced on an Illumina HiSeq 2000 (Illumina, USA). Sequencing data was processed with the Illumina pipeline v1.3.2.

Bioinformatics Analysis of sRNA Libraries from Mitochondria

All the 50 nt sequence tags generated from Illumina HiSeq 2000 went through data cleaning process which included getting rid of low quality tags. The clean tags lengths were then summarized. The clean tags were also mapped to the UCSC hg19 human assembly using SOAP (Short Oligonucleotide Alignment Program) [49] with the following options: soap -v 0 -r 2 -s 7 -p 7 -a. Sequences with perfect match or one mismatch were retained for further analysis. The presence of known miRNAs was analyzed by aligning clean tags to pre-miRNAs (1424), mature miRNAs (1539) and miRNA* (194) of miRBase 17.0 [50]. The other populations of non coding sRNAs (rRNA, scRNA, snoRNA, snRNA, tRNA and piRNA) were all identified by aligning the sequences to Genbank [51] and Rfam (www.sanger.ac.uk/resources/databases/rfam.html) using following program and parameters: blastall -p blastn -F F -e 0.01. The repeat associated RNA and piRNA were identified using tag2repeat and tag2-piRNA respectively at BGI. sRNA tags were also aligned to exons and introns of mRNA to find the degraded fragments of mRNA. All annotations were summarized using tag2annotation software in the following order of preference: rRNA (Genbank > Rfam) > known miRNA > repeat > exon > intron.

Analysis of Differential Association of miRNAs with Mitochondria of HEK293 and HeLa

To compare the differential association of miRNAs with mitochondria from HEK293 and HeLa, the miRNAs associated with mitochondria were normalized to obtain the level of association in terms of transcripts per million. If the normalized association value of a given miRNA is zero, its association value was modified to 0.01. Then, the fold-change and P-value were calculated from the normalized association using the following equations.

$$\text{Normalized association} = (\text{Actual miRNA sequencing reads count}/\text{Total clean reads count}) \times 1{,}000{,}000.$$

$$\text{Fold change} = \text{Log2}\,(\text{HEK293} - \text{NE}/\text{HeLa} - \text{NE})$$

P-value was calculated using following equation as described previously [52].

$$p(x|y) = \left(\frac{N_2}{N_1}\right)^{y} \frac{(x+y)!}{x!y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}} \qquad C\left(y \le y_{\min}|x\right) = \sum_{y=0}^{y \le y_{\min}} p(y|x) \qquad D(y \ge y_{\max}|x) = \sum_{y \ge y_{\max}}^{\infty} p(y|x)$$

The N1 and x represent total count of clean reads and normalized association level of a given miRNA in HeLa sRNA library, respectively. The N2 and y represent total count of clean reads and normalized association level of a given miRNA in HEK293 sRNA library, respectively.

Prediction of Putative Novel miRNA

All unannotated sRNA tags that aligned to UCSC hg19 were employed to screen putative novel miRNA using Mireap [http://

sourceforge.net/projects/mireap/] algorithm under these parameter settings : miRNA sequence length (18–26 nt); miRNA reference sequence length (20–24 nt); Minimal depth of Drosha/Dicer cutting site (3); Maximal copy number of miRNA on reference (20); Maximal free energy allowed for a miRNA precursor (−18 kcal/mol); Maximal space between miRNA and miRNA* (35); Minimal base pairs of miRNA and miRNA* (14); Maximal bulge of miRNA and miRNA* (4); Maximal asymmetry of miRNA/miRNA* duplex (5); Flank sequence length of miRNA precursor (10). The base bias on the first position and different position was also determined.

Clustering of miRNA Targets

The miRNAs associated with mitochondria were divided in two classes based on the number of reads: Class-I (count >5000) and Class II (count <5000). The targets were analyzed by using StarBase, a database that has been developed based on experimentally verified miRNA target interactions by CLIP-Seq (HITS-CLIP, PAR-CLIP) and degradome sequencing (Degradome-Seq, PARE) data [34]. Given that miRNA targets prediction tools give high number of false positive, only the target identified by three independent tools (PicTar, miRanda and TargetScan) and overlapped with CLIP-Seq data were taken into further consideration. The targets of putative novel miRNAs were also identified in similar way.

To characterize the biological processes regulated by targets of identified miRNAs associated with mitochondria, we used Gene Ontology (GO) and the functional annotation clustering feature of DAVID. This tool measures the similarities among related GO terms based on the extent of their associated genes and assembles the similar and redundant GO terms into annotation clusters. Fisher Exact p-value is assigned to each GO term that represents the degree of enrichment of the GO term in the input gene list. Finally, each cluster is given enrichment score to measure the biological significance. The resulting clusters were further curated to keep only GO terms with p-values >0.05. The genes with FDR≤0.05 were considered as significantly enriched in target gene candidates and were mapped in relevant signaling pathways in KEGG database [53].

Alignment of sRNAs to Mitochondrial Genome

The clean reads from both the libraries were aligned to human mitochondrial genome NC_012920 [11] through MapMi version 1.0.4.02 (EMBL) (www.ebi.ac.uk/enright-srv/MapMi) and SOAP options: -v 0 -r 2 -s 7 -p 7–a. The other populations of non-coding sRNAs (rRNA, scRNA, snoRNA, snRNA, tRNA and piRNA) were all identified by aligning to Rfam and GenBank. The alignment of putative novel miRNAs was also done by BLASTN version 2.2.20 [54] to investigate their matching sequence in mitochondrial genome.

Validation of miRNAs and their Targets Associated with Mitochondria

RNA was isolated from purified mitochondria as described above and cDNA were synthesized using Universal cDNA synthesis Kit (Exiqon, Denmark) at 42°C for 1 hr, followed by heat inactivation of enzyme at 85°C for 5 minutes. The levels of following miRNAs were determined from 10 ng cDNA using U6 snRNA (Accession number: X59362) and 5 S rRNA (NCBI Accession no: V00589) as endogenous control using miRCURY LNA™ Universal RT microRNA PCR, SYBR Green master mix (Exiqon, Denmark):

hsa-let-7b (UGAGGUAGUAGGUUGUGUGGUU);
hsa-let-7g (UGAGGUAGUAGUUUGUACAGUU);
hsa-miR-107 (AGCAGCAUUGUACAGGGCUAUCA);
hsa-miR-181a (AACAUUCAACGCUGUCGGUGAGU);
hsa-miR-221 (AGCUACAUUGUCUGCUGGGUUUC);
hsa-miR-320a (AAAAGCUGGGUUGAGAGGGCGA);
hsa-miR-145 (GUCCAGUUUUCCCAGGAAUCCCU).

The initial denaturing was done at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec followed by melt curve analysis to check the specificity.

The enrichment of target mRNAs of mitochondria-associated miRNAs was determined by qPCR using following gene specific primers: XIAP (Fwd: GCACGGATCTTTACTTTTGGG, Rev: GGGTCTTCACTGGGCTTC); MFN2 Fwd: ATGTGGCCCAACTCTAAGTG, Rev: CACAAACACATCAGCATCCAG); STAT3 (Fwd: TTCTGGGCACAAACACAAAAG, Rev: TCAGTCACAATCAGGGAAGC) as described previously.

Supporting Information

Figure S1 The genomic mapping of mitochondria-associated sRNAs. The clean sequence reads were mapped onto human reference genome (UCSC hg19) to determine the genomic locations of small RNA (sRNA) using SOAP. (A) The number of sRNA tags associated with mitochondria of HEK293 mapped at specific chromosomal position. (B) The number of sRNA tags associated with mitochondria of HeLa mapped at specific chromosomal position. The area above 0 is the number of small RNAs (sRNAs) on sense strand of chromosome, shown in blue whereas area below 0 is the number of sRNAs on the antisense strand of the chromosome, shown in red.
(TIF)

Figure S2 Classification of repeat associated reads from mitochondria-associated sRNA libraries. The total sequence reads classified as repeat associated elements were further categorized to determine the levels of each repeat associated RNAs in graphical format. (A) Detailed clustering of repeat-associated RNAs from mitochondria-associated sRNA library of HEK293. (B) Detailed clustering of repeat-associated RNAs from mitochondria-associated sRNA library of HeLa.
(TIF)

Figure S3 Association of human Argonaute with mitochondria. Argonaute proteins co-localizes with mitochondria. HEK293-MTRFP cells were transfected with pEGFPC1-Ago2, pEGFPC1-Ago3 and pAcGFP-N1. After 24 hrs of transfection, cells were stained with Hoechst and analyzed by confocal microscopy as described in Methods S1.
(TIF)

Table S1 Annotations of various classes of sRNA associated with mitochondria of HEK293. The total clean sequences obtained from the sRNA libraries were subjected to a series of sequence similarity searches using specific databases (rRNAs, tRNAs, sn/snoRNAs, miRNAs, other non-coding RNAs). The sequences that did not match with any known sequence were categorized as unannotated sequences. All annotations were summarized using tag2annotations software. An overview of sRNAs associated with mitochondria of HEK293. [1] type of sRNA, [2] total number of unique sequences belonging to each category, [3] percentage of unique sequences belonging to each category, [4] total number of all sequences belonging to each category, [5] percentage of total sequences belonging to each category.
(XLS)

Table S2 Annotations of various classes of sRNA associated with mitochondria of HeLa. An overview of sRNAs associated with mitochondria of HeLa. [1], [2], [3], [4], [5] same as table S1.
(XLS)

Table S3 Pattern of miRNAs associated with mitochondria of HEK293 and HeLa. The miRNAs associated with mitochondria from both cell lines and their respective frequency count. [1] name of miRNA according to miRBase 17.0, [2] and [3] total sequences reads that matched to particular miRNA from mitochondria-associated sRNA library of HEK293 and HeLa respectively.
(XLS)

Table S4 Putative novel miRNAs associated with mitochondria of HEK293 and HeLa. Features of putative novel miRNAs associated with mitochondria of HEK293 and HeLa as determined by miReap. [1] identification code assigned to each putative novel miRNA, [2] genomic location of each putative novel miRNA, [3] orientation of putative novel miRNA on chromosome (+/−), [4] MFE energy score (<−18 kcal/mol) of each miRNA, [5] number of sequence reads matched from the library, [6] sequence of putative novel miRNA.
(XLS)

Table S5 The GO term of predicted targets of miRNAs associated with mitochondria (HEK293 and HeLa) belonging to high frequency count category (count >5000). The targets of miRNAs associated with mitochondria (HEK293 and HeLa) belonging to high frequency count category (count >5000) were determined by StarBase and clustered into GO terms using the DAVID gene annotation tool. [1] Number of cluster and enrichment score (ES) >1.05, [2] The gene annotation term, [3] The number of target genes which belonged to GO cluster, [4] Fisher exact p-value representing the degree of enrichment of the GO term, [5] Benjamini correction value for each category.
(XLS)

Table S6 KEGG pathways enriched for targets of miRNAs associated with mitochondria (HEK293 and HeLa) belonging to high frequency count category (count >5000). The targets of miRNAs associated with mitochondria (HEK293 and HeLa) belonging to high frequency count category (count >5000) were determined by StarBase and clustered into KEGG pathways using the DAVID gene annotation tool. [1] KEGG pathway and its ID, [2] the number of target genes, which belong to the pathway, [3] Fisher Exact p-value representing the degree of enrichment, [4] Benjamini correction value for each category.
(XLS)

Table S7 The GO term of predicted targets of miRNAs associated with mitochondria (HEK293 and HeLa) belonging to low frequency count category (count <5000). The targets of miRNAs associated with mitochondria (HEK293 and HeLa) belonging to low frequency count category (count <5000) were determined by StarBase and clustered into GO term using the DAVID gene annotation tool. [1], [2], [3], [4], [5] same as table S5.
(XLS)

Table S8 KEGG pathways enriched for targets of miRNAs associated with mitochondria (HEK293 and HeLa) belonging to low frequency count category (<5000). The targets of miRNAs associated with mitochondria (HEK293 and HeLa) belonging to low frequency count category (count <5000) from were determined by StarBase and clustered into KEGG pathways using the DAVID gene annotation tool. [1], [2], [3], [4] same as table S6.
(XLS)

Table S9 The GO term of predicted targets of putative novel miRNAs associated with mitochondria of HEK293. The targets of novel miRNAs associated with mitochondria of HEK293 were determined by StarBase and clustered into GO terms using the DAVID gene annotation tool. [1], [2], [3], [4], [5] same as table S5.
(XLS)

Table S10 KEGG pathways enriched for targets of putative novel miRNAs associated with mitochondria of HEK293. The targets of putative novel miRNAs associated with mitochondria of HEK293 were determined by StarBase and clustered into KEGG pathways using the DAVID gene annotation tool. [1], [2], [3], [4] same as table S6.
(XLS)

Table S11 The GO term of predicted targets of putative novel miRNAs associated with mitochondria of HeLa. The targets of putative novel miRNAs associated with mitochondria of HeLa were determined by StarBase and clustered into GO term using the DAVID gene annotation tool. [1], [2], [3], [4], [5] same as table S5.
(XLS)

Table S12 KEGG pathways enriched for targets of putative novel miRNAs associated with mitochondria of HeLa. The targets of putative novel miRNAs associated with mitochondria of HeLa were determined by StarBase and clustered into KEGG pathways using the DAVID gene annotation tool. [1], [2], [3], [4] same as table S6.
(XLS)

Methods S1 Supplementary Methods.
(DOC)

Acknowledgments

We thank Dr. Jassie Cameron (Dept. of Genetics and Genome Biology, Hospital for Sick Children, Toronto, Canada), Mikael S. Lindström (Department of Oncology-Pathology, Cancer Center Karolinska, Karolinska University Hospital in Solna, Stockholm, Sweden) for providing rabbit polyclonal NDUFS2, RPS9 and cytochrome c antibody respectively. We also thank Jose Afonso Guerra-Assunção (European Bioinformatics Institute, Welcome Trust Genome Campus, Hinxton, Cambridge, UK) for assistance in MapMi alignments. pEGFPC1-Ago2/3 was kind gift of Dr. Hank Qi (Department of Anatomy and Cell Biology, Carver College of Medicine, University of Iowa).

The raw sequence data from this study have been submitted to Gene Expression Omnibus under accession nos. GSM797669 and GSM797670.

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=rxkpzoiwucsykty&acc=GSE32185.

Author Contributions

Conceived and designed the experiments: LS Rochika Singh. Performed the experiments: LS DT. Analyzed the data: LS DT Rochika Singh Rajesh Singh. Contributed reagents/materials/analysis tools: PP AKS Rochika Singh. Wrote the paper: LS DT Rajesh Singh.

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J Mol Med
DOI 10.1007/s00109-017-1517-5

JMolMed CrossMark

ORIGINAL ARTICLE

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

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Received: 27 June 2016 / Revised: 7 January 2017 / Accepted: 7 February 2017

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

Electronic supplementary material The online version of this article (doi:10.1007/s00109-017-1517-5) contains supplementary material, which is available to authorized users.

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Published online: 20 February 2017

proteins [13–15]. The encoding capacity of human mitochondrial DNA is limited to 13 proteins, 16S rRNA, 12S rRNA, and 22 tRNAs. Thus, most of the mitochondrial proteins are encoded by the nuclear genome, translated in the cytoplasm, and then transported to mitochondria. Emerging evidence suggests that mRNAs and noncoding RNAs (ncRNAs) transcribed from the nuclear genome are also transported to mitochondria where they are required for their optimal function. Rates of RNA and protein transport to mitochondria are cell type specific and depend upon the specific energy demands [16].

It has previously been observed that noncoding RNAs, specifically miRNAs, associate with different subcellular organelles [17], although their impact on cellular functions is not well understood. miRNA at these sites may regulate the copy number of target mRNA to keep protein levels in a narrow physiological range to ensure optimal function of the associated organelles. Previous reports from our lab and others [17, 18] demonstrated the association and import of ncRNAs including miRNAs into mitochondria. Interestingly, miR-4485, one of the miRNAs identified in our recent systematic analysis of mitochondrial miRNAs, is homologous to the 16S rRNA region in the mitochondrial genome [19]. In the present study, we analyze the association and transport of miR-4485 into mitochondria, where it regulates the processing of mitochondrial 16S rRNA and modulates the bioenergetic status. The expression of miR-4485 affects growth of breast cancer cells in cell culture and in a mouse xenograft model.

Materials and methods

Cells and cell culture

HEK293, HeLa, and MCF-7 cells were cultured in Dulbecco's modified Eagle's media (DMEM) (Life Technologies, USA) supplemented with 10 % (*v*/v) heat-inactivated fetal bovine serum (Life Technologies, USA), 1 % penicillin, and streptomycin and neomycin (PSN) antibiotic mixture (Life Technologies, USA). The cells were maintained in 5 % CO_2 at 37 °C. The mitochondrial DNA-depleted HEK293 cells were prepared and maintained as described previously [20]. MDA-MB231 cells and their derivatives were grown on DMEM supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 100 U/ml streptomycin, and 100 U/ml penicillin.

Patient tissue/ethics statement

Human breast tumor and extra-tumoral tissue specimens were collected from patients undergoing mastectomy. Human studies were performed following the norms of 1964 Declaration of Helsinki and approved by the ethical committee of Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, UP, India. The consent of each patient was obtained prior to tissue collection. 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 was frozen in liquid nitrogen and then stored at −80 °C for RNA extraction.

RNA isolation and expression analysis

RNA was isolated from total cells, mitochondria, mitoplast, and RNaseA-treated mitochondria as described previously [19]. The putative targets of hsa-miR-4485 were analyzed in starBase v.2.0 [21] by feeding seed sequence of miR-4485. The binding partners of miR-4485 were determined by RNA immunoprecipitation using biotin-tagged miR-4485 [22] as detailed in SI "Materials and methods."

Mitochondrial assays

miR-4485 mimic/inhibitor was transfected and lactate dehydrogenase (LDH) activity, mitochondrial DNA-encoded RNA processing, translation pattern, ATP levels, ROS generation, complex I activity, and mitochondrial membrane potential were determined as detailed in SI "Materials and methods."

Cell viability and tumorigenicity

miR-4485 mimic was transfected and cellular viability was determined by MTT assay, apoptosis by caspase-3/7 luciferase activity assay, and tumorigenic potential by clonogenic assay and in vivo xenograft model in nude mice as detailed in SI "Materials and methods."

Statistical analysis

The data are represented as SEM of two to four independent experiments. The data sets were normalized considering the values of controls as 100 %. The comparisons between data sets were performed by unpaired two-tailed Student's *t* test using GraphPad Prism 5. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ were considered to be statistically significant.

Results

Nuclear-encoded miR-4485 translocates to mitochondria

In eukaryotic cells, mitochondria are known to import nuclear-encoded cytosolic RNAs [17], although the transport

of miRNAs to mitochondria is poorly studied. Previously, we identified miR-4485 as one of the human miRNAs associated with mitochondria [19]. A sequence analysis showed that miR-4485 maps to the intronic region of the MTRNR2L8 gene on chromosome 11 (Fig. S1A) and its sequence aligns within the 16S rRNA region of the mitochondrial genome (Fig. S1B). 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 (Fig. 1a). 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 [19] as a negative control for mitochondrial association (Fig. 1a). RNaseA treatment of purified mitochondria did not eliminate the association of miR-4485 (Fig. 1b) 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 (Fig. 1c). The results indicate that miR-4485 is present inside mitochondria.

In the cytosol, pre-miRNAs are processed by Dicer to form the mature miRNA [23]. 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 [24, 25]. Our results show high levels of precursor miR-4485 in the cytosol (Fig. 1d) but low levels in mitochondria, suggesting that the precursor miR-4485 is present in the cytosol, while its mature form associates with mitochondria.

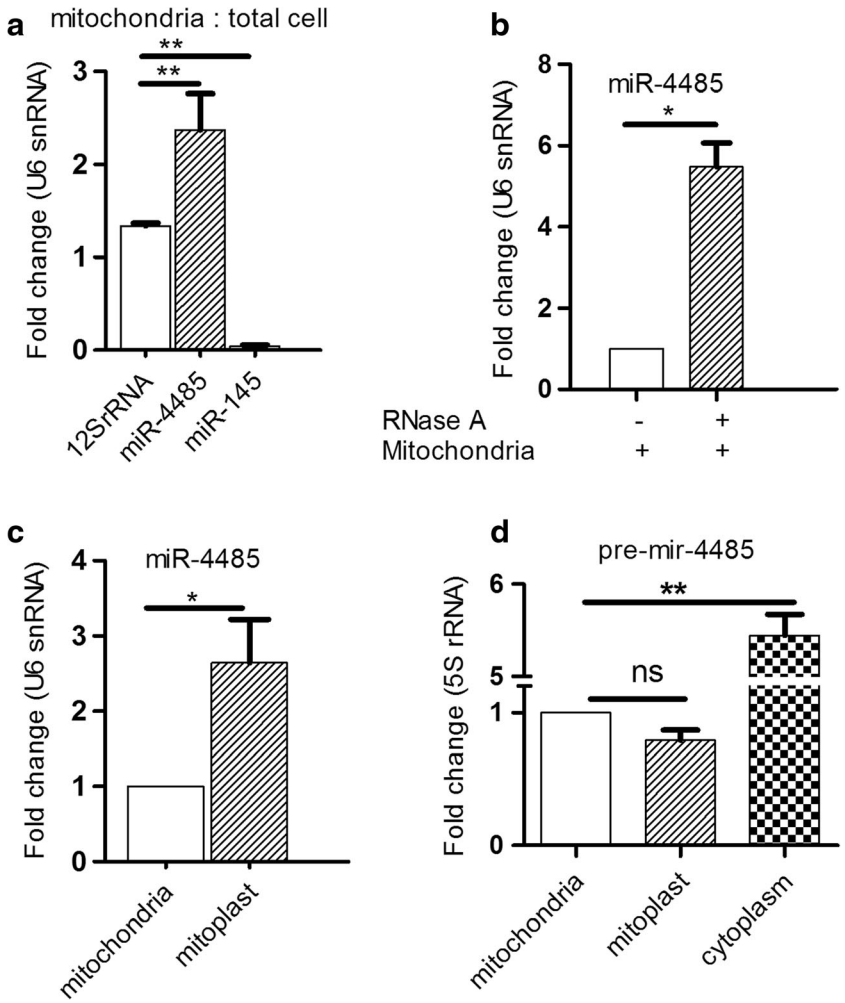

Fig. 1 miR-4485 associates with human mitochondria. **a** Small RNA was extracted from total cell and mitochondria of HEK293, polyA tailing was performed, and cDNA was synthesized. The relative association of 12S rRNA, miR-4485, and miR-145 was determined using U6 snRNA as endogenous control by qPCR. **b** Mitochondria were isolated from HEK293 and treated with RNaseA to remove nonspecific 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. $^{*}p < 0.01$; $^{**}p < 0.001$

Expression levels and subcellular localization of specific miRNAs may vary upon diverse pathophysiological conditions [17, 26–29]. We found that the level of miR-4485 in mitochondria increases after the treatment with H_2O_2 (Fig. S2A) and TNF-α (Fig. S2B), whereas it decreases during ER stress (Tunicamycin) (Fig. S2C) and mitochondrial stress (Rotenone) (Fig. S2D). The results suggest a dynamic association of miR-4485 with mitochondria under different stress conditions.

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

Recently. it was suggested that miR-4485 may represent a processing product of ASncmtRNA-2, alncRNA derived from the 16S rRNA region of the mitochondrial genome [30]. 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, prepared by repeated passaging in the presence of ethidium bromide [20, 31]. The levels of mitochondrial DNA-encoded ND4 and CYB gene transcripts were significantly decreased in ρ0 cells (Fig. 2a). Similarly, levels of miR-4485 were decreased in ρ0 cells as compared to control cells (Fig. 2b). As the genomic locus of miR-4485 maps to chromosome 11, the nuclear origin of miR-4485 was tested by inhibiting the nucleocytoplasmic 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 [32]. Treatment of HEK293 cells with Leptomycin B decreased the level of miR-4485 in mitochondria (Fig. 2c). We also tested effects of inhibitors of canonical miRNA biogenesis and processing. Treatment of cells with trypaflavine (TPA), a chemical inhibitor of mitochondrial transcription and Argonaute2 binding, showed no change in the level of mature miR-4485 in mitochondria. Interestingly, treatment with poly-L-lysine (PLL), an inhibitor of Dicer, substantially decreased the level of miR-4485 in the mitochondrial fraction (Fig. 2d). Ectopic overexpression of Dicer showed an increased level of miR-4485 in the mitochondrial fraction (Fig. 2e). These results suggest that

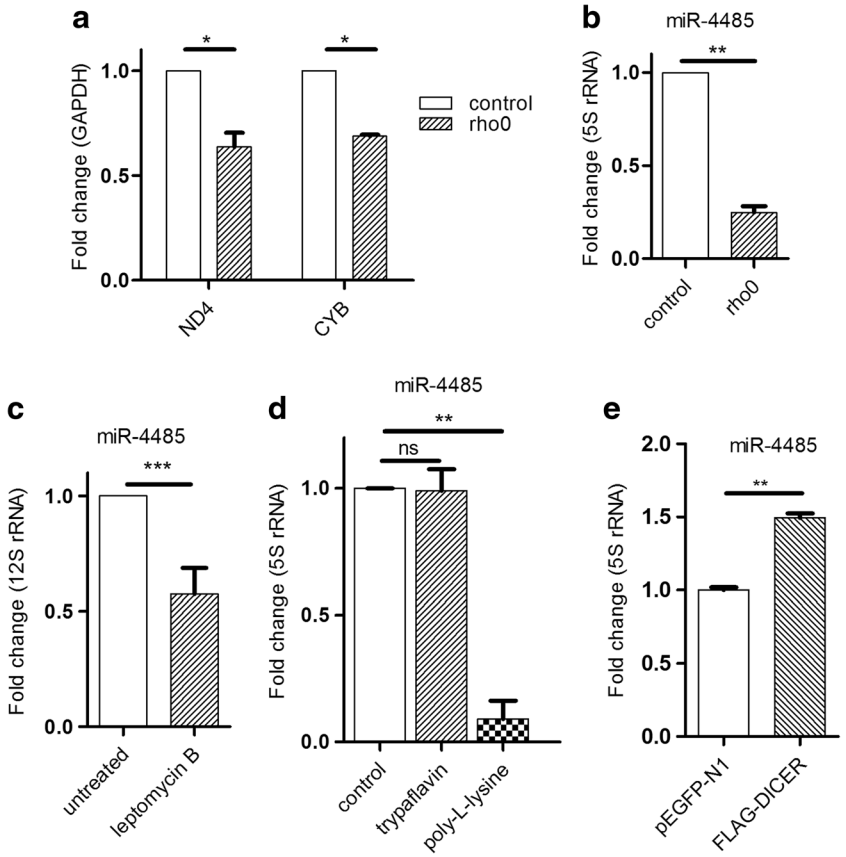

Fig. 2 Nuclear transport and processing are required for association of miR-4485 with mitochondria. **a** The levels of mitochondrial DNA-encoded transcripts, ND4 and CYB, were analyzed in control vs rho0 cells by qPCR. **b** The relative quantification of miR-4485 was performed by qPCR in same sets. **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 trypaflavine and poly-L-lysine. The level of miR-4485 was determined by qPCR. **e** HEK293 cells were transfected with pEGFP-N1 and Flag-Dicer and levels of miR-4485 were determined. $^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$

miR-4485 is encoded by the nuclear genome, processed in the cytoplasm by Dicer to be accumulated in mitochondria.

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

To further analyze the role of miR-4485 in the 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 [33]. 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 (Fig. S3A) 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 (Fig. 3a). 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 downregulation of all mature mRNAs (Fig. 3b). A direct binding of miR-4485 to mitochondrial 16S rRNA was confirmed by RNA immunoprecipitation using biotin-labeled miR-4485. 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 (Fig. 3c). miR-4485 interferes with the biogenesis of mitochondrial 16S rRNA, hence it may also affect the translation of mitochondrial DNA-encoded proteins. Therefore, we monitored the level of mitochondrial DNA-encoded proteins by incorporation of the methionine homolog L-azidohomoalanine (AHA), followed by conjugation with biotin [34]. The expression of miR-4485 decreased the level of all proteins encoded by the mitochondrial genome (Fig. 3d). The results suggest that miR-4485 interferes with 16S rRNA processing and decreases mitochondrial protein synthesis.

miR-4485 regulates mitochondrial functions

As the mitochondrial genome encodes subunits of respiratory complexes, the downregulation 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 (Figs. 4A and S3B) and mitochondrial (Fig. 4b) ATP levels decreased in cells transfected with miR-4485 mimic. The transfection of miR-4485 mimic decreased the activity of mitochondrial respiratory complex I (Fig. 4c). Complex I dysfunction is known to be associated with the increased production of ROS [35]. Interestingly, we observed both elevated ROS levels (Figs. 4D and S3C) and a decreased mitochondrial membrane potential (Figs. 4E and S3D) 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 [36]. The transfection of miR-4485 mimic decreased the cell viability (Fig. 4f) and increased the caspases-3/7 activity (Fig. 4g), which is a hallmark of apoptosis induced by mitochondrial dysfunction [37]. 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 (Fig. S4A) and ROS levels (Fig. S4B) and improved mitochondrial membrane potential (Fig. S4C). The inhibitor decreased caspase-3/7 activity (Fig. S4D) and prevented cell death (Fig. 4f).

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

To further understand pathophysiological implications of miR-4485, we looked for potential targets of miR-4485 by using StarBase v.2.0 tool, which provides high-confidence target predictions (Table S3). Interestingly, most of the identified putative targets of miR-4485 have 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 (Fig. S5A) while the opposite was observed in the presence of miR-4485 inhibitor (Fig. S5B).

Alterations of mitochondrial functions and reprogramming of energy metabolism represent hallmarks of cancer [5, 6]. Aerobic glycolysis plays an essential role in the bioenergetics of cancer cells [7]. To further understand the role of miR-4485 in tumorigenesis, we monitored markers of the glycolytic shift, LDH activity, and clonogenic potential (shown as

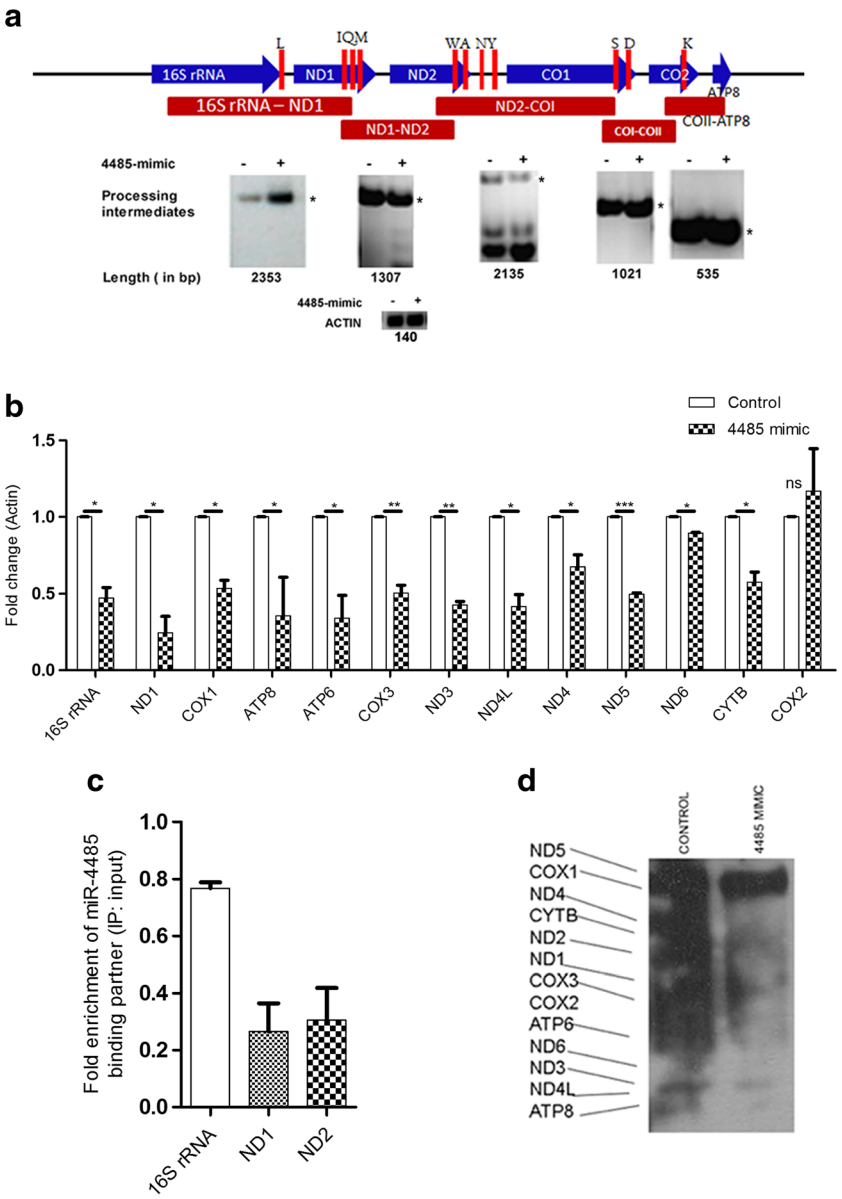

Fig. 3 miR-4485 regulates mitochondrial RNA processing and translation. **a** The role of miR-4485 on mitochondrial processing was analyzed as described in "Materials and methods." Locations of mitochondrial DNA-encoded specific transcripts (*blue arrows*) and tRNAs (*pink marks*) have been shown. Positions of the PCR amplicons were analyzed and 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 were determined by qPCR in HEK293 cells. **d** The effect of miR-4485 on mitochondrial protein synthesis was determined by AHA incorporation and captured by biotin using Click-iT Labeling kit (Thermo-Fisher) in MCF-7 cells. $^{*}p < 0.01$, $^{***}p < 0.0001$

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 (Fig. 5a) as compared to control. Further, transfection of miR-4485 mimic and Cy5-miR-4485 decreased the LDH activity (Fig. S6A). The transfection of miR-4485 mimic in MCF-7 and MDA-MB-231 breast cancer cell lines decreased the clonogenic potential (Figs. 5B and S6B/C). The transfection of miR-4485 inhibitor in MCF-7 had an opposite effect (Fig. S6D). As we observed here, miR-4485 regulates ROS levels (Figs. 4D and S3C); hence, we analyzed the clonogenic potential of breast cancer cells in the presence of ROS scavenger *N*-acetyl-L-cysteine (NAC). Cell survival (Fig. 5C) and plating efficiency (Fig. 5D) significantly increased in miR-4485 mimic-transfected MCF-7 cells in the

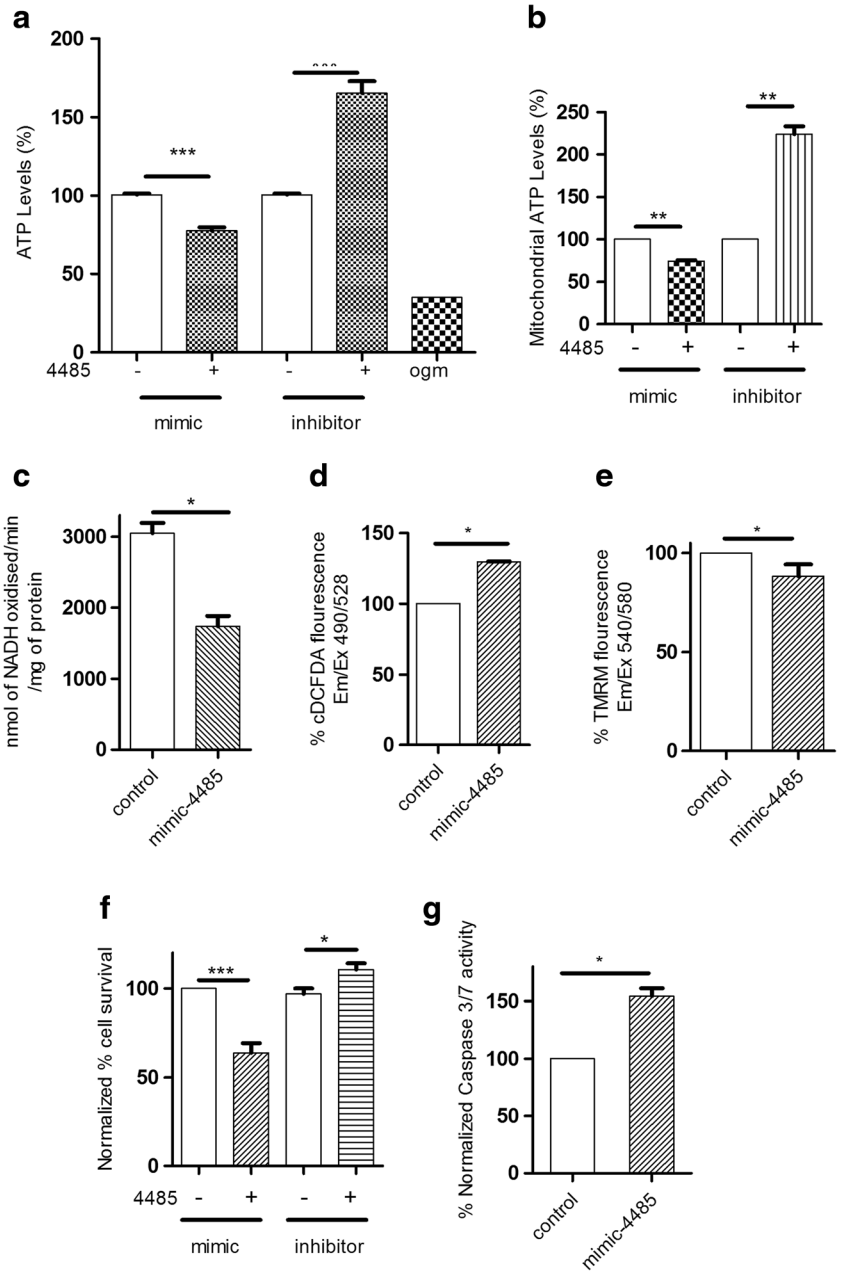

Fig. 4 miR-4485 regulates mitochondrial function, ROS, and cell death. The mimic of miR-4485 was transfected along with control in HEK293 and effect on mitochondrial functions was analyzed. **a** Levels of total cellular ATP (oligomycin was added as control) and **b** mitochondrial ATP were measured using ATP detection kit. **c** The mitochondrial complex I activity was measured as reduction of NADH per minute per milligram 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. $^{*}p < 0.01$, $^{***}p < 0.0001$

presence of NAC. Similar results were observed in the cervical carcinoma cell line HeLa (Fig. S6D). These results suggest that miR-4485 may downregulate glycolysis and decrease the clonogenic potential of breast cancer cells.

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 a miR-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 (Fig. 5e). We then analyzed the expression of miR-4485 in breast cancer

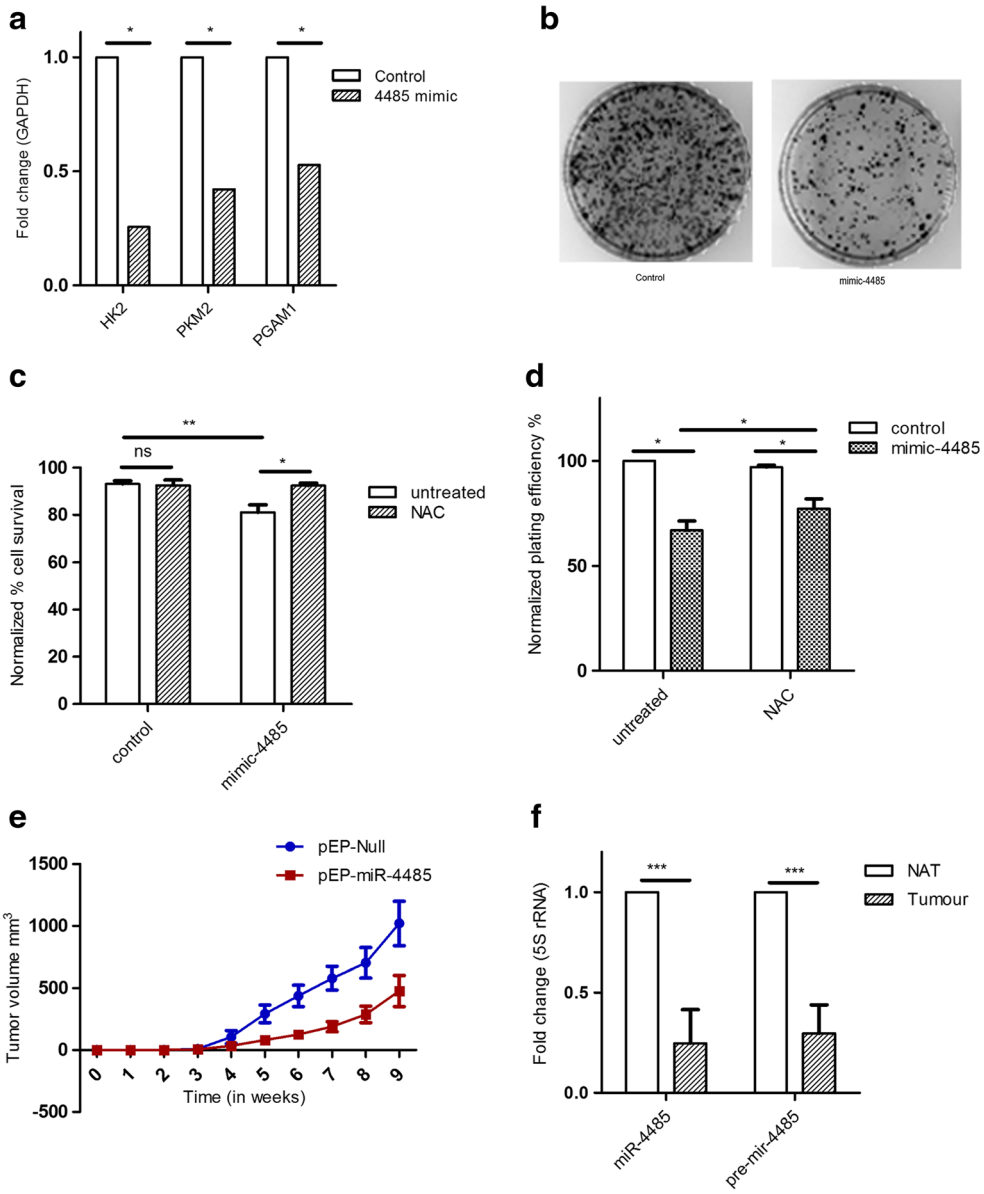

Fig. 5 miR-4485 negatively regulates tumorigenic potential of breast cancer cells. **a** The total cellular RNA was prepared from MCF-7 cells transfected with mimic. The relative expression of the genes regulating glycolysis was analyzed by real time PCR. **b** The mimic of miR-4485 was transfected in MCF-7 cells and clonogenic assay was performed. To further elucidate the role of ROS in tumorigenesis, MCF-7 were 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 (MDA-MB-231) 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 breast tumor and non tumoral adjacent tissue was determined by qPCR ($n = 7$). $^{*}p < 0.01$, $^{***}p < 0.0001$

patients. Levels of both mature miR-4485 and its precursor transcript were found decreased in seven breast tumor tissues, as compared to the non-tumoral adjacent tissue (NAT) (Fig. 5f). These results indicate that mitochondrial targeted miR-4485 negatively regulates tumorigenicity in breast cancer.

Discussion

The regulation of mitochondrial function is critical for maintaining cellular and tissue homeostasis. 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. Replication of the mitochondrial genome, transcription, and expression of its transcripts is coordinated by nuclear genome-encoded proteins and RNAs that are imported to mitochondria [38]. Previous reports from our group [19] 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, alncRNA derived from the 16S rRNA region of the mitochondrial genome [30]; 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 the miR-4485 precursor transcript was enriched in the cytosol. The inhibition of Dicer activity decreased the level of miR-4485 in mitochondria. Also, the inhibition of TRBP-Ago2 interaction by trypaflavine 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 translocated to mitochondria. However, we cannot rule out the possibility that a sub-fraction of miR-4485 originates from the mitochondrial genome.

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 miR-4485 mitochondrial translocation. Transfection of miR-4485 mimic showed decreased levels of mitochondrial transcripts. The mitochondrial genome is expressed as a precursor transcript, which is further processed to mature mRNAs by a rather complex process [39–42]. Our results 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 [40–42]. 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 [18] 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 [43]. Indeed, the miR-4485 mimic affects mitochondrial transcripts, which downregulate 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 [44] 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 also be 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 levels by miR-4485 suggests its participation in metabolic reprogramming of cancer cells [6, 8]. 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 [45–47]. 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 a major source of energy and anaplerotic replenishing TCA cycle with intermediate metabolites for rapid proliferation [10]. 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 mouse 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 antitumor effect. 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 need 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.

Acknowledgements This work is being supported by EMR/2014/000219, DST, Government of India to RS. This work is lead obtained from previous DBT grant BT/PR12906/AGR/36/638/2009, DBT, Government of India. A.V.L. and P.M.C. were supported by the grant 14-50-00060 from the Russian Science Foundation. The authors acknowledge the instrumentation facility by DBT MSUB ILSPARE. We thank Prof. Narry Kim, Institute for Basic Science and Seoul National University, Korea for giving us plasmids of pCK-Flag-Dicer as a gift.

L.S., A.S., and K.S. received their Senior Research fellowship from University Grants Commission (UGC), Government of India. K.B. received her Senior Research fellowship from the Council of Scientific and Industrial Research (CSIR), Government of India. Dr. Rochika Singh received her fellowship as DST Young Scientist from Department of Science and Technology (DST), Government of India. This work consists of the PhD thesis of L.S.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest related to the study.

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Mitochondrion 12 (2012) 593–599

Contents lists available at SciVerse ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

Review

Mitochondria: One of the destinations of miRNAs

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ARTICLE INFO

Article history:
Received 16 February 2012
Received in revised form 22 August 2012
Accepted 9 October 2012
Available online 17 October 2012

Keywords:
Mitochondria
RNA trafficking
Small RNA
miRNA

ABSTRACT

The cellular processes are controlled by a narrow range of mRNA and proteins levels, where small RNAs (sRNAs) known as miRNAs play a critical role. The spatial and temporal regulation of miRNA processing components and mature miRNA is emerging. The recent studies suggest that mitochondria are one of the destinations of pre as well as mature miRNAs. The role of mitochondria extends beyond energy metabolism to many other cellular processes like metabolism, cell death and inflammation. The new found destination of miRNAs suggest the role of mitochondria in monitoring site specific regulations of proteins as well as the function of mitochondria. The studies in this direction will decipher the novel role of mitochondria-associated miRNAs in different cellular processes. This review is focussed on the recent studies demonstrating the presence of miRNAs in mitochondria and its possible significance in different cellular and physiological conditions.

Contents

1. Introduction

Mitochondria are one of the central organelle involved in cellular energy metabolism. There are several examples where mitochondria play regulatory role in many human pathologies ranging from inborn errors of metabolism, cancer, inflammation and infections (Chatterjee et al., 2011; Cloonan and Choi, 2011; Dimauro and Garone, 2011; Tschopp, 2011). The dysfunction of mitochondria leads to numerous unrecognizable pathologies like neuro-muscular degeneration, cardiomyopathies and cancers (Duncan, 2011; Schon and Przedborski, 2011). Mitochondria has been the focus of intense research during the last decade however the more we understand the more we reveal mysteries of this organelle.

Mitochondria are known to play a critical role in many cellular processes like cell death, autophagy, metabolic pathways and fatty acid oxidation, Ca^{2+} homeostasis and ageing (Pan et al., 2011; Weber and Reichert, 2010). The regulation of mitochondrial function is complex and critically determined by proteins encoded from both nuclear and mitochondrial genome. The genetic information flows according to central dogma (from DNA to mRNA to proteins) (Cooper, 1981). However, emerging evidence suggest that there are different regulators at each step of central dogma. The excess or down regulation of any protein

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http://dx.doi.org/10.1016/j.mito.2012.10.009

from its normal physiological levels are undesirable for the normal physiology of cell. Hence, the fine-tuning of mRNA and protein levels in the normal physiological range is essential for cell survival during normal and stress conditions. One such key regulators of mRNA and protein levels in physiological as well as pathological conditions are small non-coding (nc) RNA molecules called as miRNA (Ambros, 2001). We discuss here the biogenesis and spatio-temporal regulation of miRNAs in mitochondrial context and its physiological impact.

2. Biogenesis of miRNA

The 1000 nucleotide (nt) long primary miRNA (pri-miRNA) is transcribed from genomic DNA like mRNA by RNA polymerase II (Lee et al., 2002). However, few subsets of miRNA are also processed by RNA polymerase III (Borchert et al., 2006). The pri-miRNA is then processed by Dorsha/Pasha microprocessor complex into ~70 nt precursor miRNA (pre-miRNA) with a 5′ phosphate and a 2-nt 3′ overhang (Denli et al., 2004; Gregory et al., 2004). The 2-nt overhang of pre-miRNA is recognized by Exportin-5 and exported to cytosol in Ran-GTP dependent manner (Lund et al., 2004; Yi et al., 2003). In the cytosol, pre-miRNA is cleaved by Dicer about two helical turns away from the ends of the stem loop, to form ~20 nt miRNA/miRNA* duplex. The double stranded miRNA is embedded into miRISC complex (Bernstein et al., 2001; Martinez et al., 2002; O'Carroll and Schaefer, in press) consisting of Dicer, Argonaute and RNA-binding proteins TRBP and PACT (Gregory et al., 2005; Kok et al., 2007). The miRNA duplex is unwound to form single-stranded mature miRNA (the guide strand) (Nykanen et al., 2001) while its complementary strand (the passenger strand or miRNA*) is degraded by miRISC complex (Leuschner et al., 2006; Matranga et al., 2005; Rand et al., 2005). The guide strand of miRNA binds to the complimentary region of target mRNA. This miRNA/mRNA complex is recognized by Argonaute protein, and glycine–tryptophan protein of 182 kDa (GW182). These proteins act as key factors in the assembly of miRISC and governs the process of translation repression or degradation of target mRNA (Fig. 1). The mechanism of translation repression is tightly regulated during different cellular and pathological conditions to fine tune the levels of mRNA/proteins. The miRNA levels itself are tightly regulated starting from biogenesis to maturation (Iorio and Croce, 2012; Yang and Wang, 2011). The regulation of miRNA levels can be temporal or spatial.

Interestingly, the localization of pre and mature miRNA and proteins involved in its biogenesis are not confined to their originally assumed site of cellular localization (cytosol). They are enriched in various sub-cellular compartments: nucleus (Buchan and Parker, 2007), processing (P) bodies (Huang et al., 2011; Xie et al., 2007), Golgi bodies, Endoplasmic Reticulum, Multiple Vesicular Bodies (MVB) and exosomes (Gibbings, 2011; Lee et al., 2009). Recently it has been observed that miRNAs are enriched in/associated with mitochondria (Bandiera et al., 2011; Barrey et al., 2011; Bian et al., 2010; Kren et al., 2009; Mercer et al., 2011; Sripada et al., 2012).

The trafficking of nuclear encoded protein to mitochondria and other cellular compartments has been an intense area of research and is well understood whereas the trafficking of RNA to different sub-cellular compartments including mitochondria and their physiological and pathological implication is beginning to emerge.

3. Trafficking of RNA across mitochondrial membrane

The mitochondria contain ~1500 proteins while its genome encodes only 13 proteins, hence the other nuclear encoded proteins are imported into mitochondria for the optimum function (Lopez et al., 2000). The mitochondrial targeted proteins encoded from nuclear genome are imported through TOM/TIM complexes (outer and inner mitochondrial membrane translocation proteins) with the help of numerous accessory proteins. The transport occurs either by presequence recognition pathway, carrier protein transport pathway, redox-regulated import

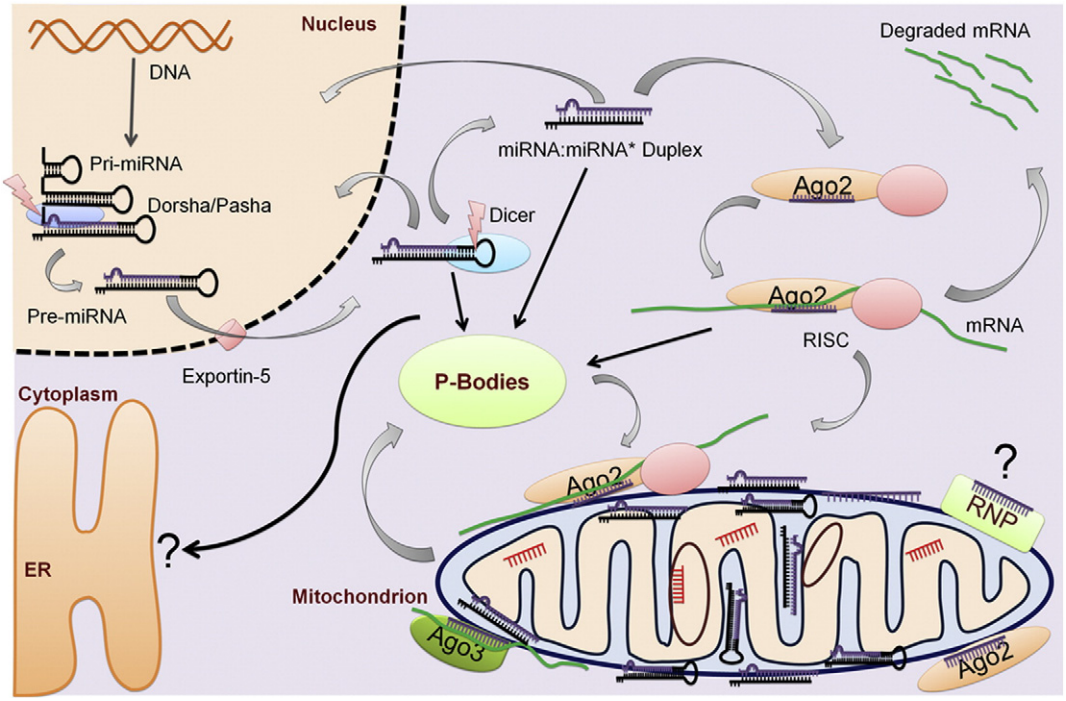

Fig. 1. Spatial regulation of miRNA in human cell. The pri-miRNA is transcribed from genome by RNA polymerase II. The pri-miRNA is processed into pre-miRNA by RNase III enzyme Dorsha with the help of its partner Pasha. The 3′ nt overhang is recognised by Exportin-5 which exports the pre-miRNA into the cytosol in Ran-GTP dependent manner. In cytosol pri-miRNA is cleaved by Dicer to produce mature miRNA which then becomes a part of RISC assembly to attenuate or degrade complimentary mRNA. pre-miRNA and mature miRNA are translocated to various subcellular locations (nucleus, ER, P-bodies, etc.) including mitochondria. The pre-miRNA, mature miRNA and core component of RISC, Ago2/3 are associated with mitochondria. The store house of miRNA, P-bodies is also associated with mitochondria. Mitochondrial outer membrane may serve as a platform for the assembly of miRNA and its processing components. miRNA may also translocate to the mitochondrial matrix to regulate mitochondrial gene expression. The sequences of few miRNA aligned to mitochondrial DNA.

pathway, β-barrel pathway or by oxidative folding pathway (Dudek et al., in press; Mesecke et al., 2005). These pathways have been studied for the last several decades and have been reviewed extensively (Becker et al., 2012; Chacinska et al., 2009; Harsman et al., 2011; Herrmann and Riemer, 2012; Voos, in press). Similarly, RNA import is crucial for mitochondrial function, however the mechanisms regulating RNA import have still not been well investigated.

It is well known that mitochondria imports nuclear encoded tRNA (Alfonzo and Soll, 2009; Duchene et al., 2009). The number of tRNA imported into mitochondria ranges from one in yeast, to all in trypanosomes. The tRNA import in yeast occurs by both protein import machineries (TOM-TIM complex) and non canonical import systems (Rubio et al., 2008). Mammalian mitochondria are known to import/export tRNA both *in vitro* and *in vivo* (Kolesnikova et al., 2004; Rubio and Hopper, 2011). Interestingly, the mitochondrial tRNAMet, tRNAIle, tRNALys, and tRNAGlu were found in the cytosol (Maniataki and Mourelatos, 2005). However, the significance of this finding is yet unknown. The other essential nuclear encoded ncRNAs transported into mitochondria are component of mitochondrial endoribonuclease, RNase MRP (which processes mitochondrial leading strand transcript to form primers for DNA replication) and RNase P (which cleaves polycistronic transcripts to generate individual mtRNAs and processes 5′ end of tRNA) (Puranam and Attardi, 2001; Wang et al., 2012a). Similarly, the nuclear-encoded 5S rRNA is the most abundant RNA in mitochondria (Entelis et al., 2001; Smirnov et al., 2008) which is a crucial component of mitochondrial ribosomal assembly. The import of 5SrRNA was recently found to be dependent on mitochondrial enzyme rhodanese (thiosulfate sulfurtransferase), which interacts with both α-domain and γ-domain regions of 5S rRNA and mitochondrial ribosomal protein L18 (MRP-L18), a member of the L18/eL5 family(Smirnov et al., 2011). The mechanisms of back and forth transport of RNA from mitochondria is emerging; however their significance in context of small RNA specifically miRNA still needs to be understood.

4. Small RNA in mitochondria

The systematic analysis of sRNA in organelles having their own genome specifically mitochondria and chloroplast was first initiated by Lung in 2006. It was observed that the majority of the ncRNA present both in mitochondria and chloroplast were not encoded by their own genome and postulated to be imported from nucleus (Lung et al., 2006). The emerging role of small RNA and mitochondria in many patho-physiological conditions has led to focus recent studies of ncRNA and their possible association with mitochondria. The whole mitochondrial transcriptome from different human cell lines demonstrated the presence of nc-RNA (piRNA, snRNA, snoRNA, srpRNA and miRNA) associated with mitochondria (Mercer et al., 2011). Interestingly, deep sequencing of mitochondrial associated sRNA revealed the association of a diverse population of ncRNAs (miRNA, piRNA, snRNA, snoRNA, srpRNA and repeat associated RNA elements) with mitochondria of HEK293 and HeLa (Sripada et al., 2012).

It has been observed that Zucchini, a cytoplasmic nuclease essential for pri piRNA processing, is localized in mitochondria. The close association of nuage bodies with mitochondria in *Drosophila* (Aravin and Chan, 2011) suggest the active role of mitochondria in biogenesis and regulation of piRNA. This active crosstalk may also be the reason for the observed presence of piRNA in mitochondria. The significance of association of piRNA with mitochondria is not clear and its role in different cellular processes needs to be further studied.

The direct explanations of snRNA, srpRNA and repeat associated RNA enrichment in mitochondria are still lacking. However, the small non coding RNA (7-2/MRP RNA) component of MRP is enriched both in nucleolus and mitochondria. In mitochondria it generates primers for mitochondrial DNA replication by cleaving the RNA transcribed from the mitochondrial leading strand whereas in the nucleus it associates with the 80S subunit of ribosomes and is involved in pre-mRNA processing (Kiss et al., 1992). These evidences suggest that snRNA may localize in different cellular compartments and show distinct functions. Similarly, snoRNA (small nucleolar RNA) modulates the maturation of rRNA and snRNA in nucleolus (Matera et al., 2007). It was observed recently that biogenesis of miRNA may take up non-canonical pathways involving cleavage from tRNA or snoRNAs leading to sRNA similar to miRNA. (Yang and Lai, 2011). For instance, snoRNA (ACA45) is cleaved by dicer, associates with Ago2 in RISC complex and targets CDK11 (Ender et al., 2008; Yang and Lai, 2011).

However, such RNAs are not recognised as miRNA by the sRNA annotating programs. Hence the clear cut demarcation of sRNA and their importance in mitochondrial enrichment needs demonstration. These evidences suggest the cross talk of tRNA and miRNA. There are possibilities that miRNA biogenesis is at/near mitochondria through tRNA/snoRNA cleavage or are the actual experimental contaminants. The association of sRNA with mitochondria needs to be considered keeping the experimental errors in mind. The degradation product sRNA having a sequence similar to miRNA may result in misleading artefacts. It can be avoided by ensuring proper handling and validating the same experiments repeatedly using different methods. The association of other small RNA with mitochondria is beyond the focus of the current review however it is an interesting and emerging area for further investigation.

4.1. miRNA in or associated with mitochondria?

miRNA plays an essential role in modulating gene expression by binding to complementary sites on the target mRNA, thereby blocking translation or degrading the target mRNA (Sun et al., 2011). The dysregulation of miRNA biogenesis and maturation may have serious repercussions in different cellular processes. Since mitochondria are important subcellular organelles that contain proteins mostly encoded from nuclear genome (Fernandez-Silva et al., 2003), hence the organelle may be the potential site for miRNA-mediated post-transcriptional regulation.

Recently miRNAs have been found to be present in mitochondria isolated from rat liver (15 miRNAs), mouse liver (20 miRNAs), myotubes (20 miRNAs), HeLa (6 miRNAs), 143B (3 miRNAs), HEK293 (6 miRNAs) and human muscles (46 mature miRNAs and 2 pre-miRNAs) (Table 1, Table S1) (Bandiera et al., 2011; Barrey et al., 2011; Bian et al., 2010; Kren et al., 2009; Mercer et al., 2011; Sripada et al., 2012). Interestingly, the nuclear encoded mRNA and miRNAs were recently found to be associated with outer mitochondrial membrane and are depleted in mitoplast (Mercer et al., 2011; Sripada et al., 2012). This implies that miRNAs are more significantly associated with mitochondria rather than their enrichment inside the double membrane bound organelle. However, the dynamics and enrichment of miRNAs in various submitochondrial compartments have to be determined. The outer mitochondrial membrane may provide platform to assemble novel signalling complexes, which play an important role in regulation of transcriptional repression. This hypothesis needs experimental verifications. The supporting evidences suggest that Ago2 and Ago3, key components of miRISC complex co-localises with mitochondria (Barrey et al., 2011; Sripada et al., 2012). This raises question of association of other RISC members with mitochondria, serving as platform for transcriptional repression platform like CLPABP-RNA protein complex. The CLPABP (Cardiolipin and phosphatidic acid binding protein) is a RNA granule comprising RNA binding/stabilising/metabolic proteins, localized on a tubulin network with close proximity to mitochondria. This complex is unique as it binds to cytochrome c mRNA, regulates its transport and its translation by regulating its copy number through another protein HuR, having RNase activity (Sano et al., 2008). Similarly mitochondria have also been observed to dynamically associate with P bodies (Ernoult-Lange et al., 2012) and stress granules (Cande et al., 2004), the cytoplasmic bodies which are the hub of miRNAs, its precursors and biogenesis components. These unique examples suggest that outer

Table 1
miRNA associated with mitochondria.

Source of mitochondria[1]	miRNAs[2]
mouse liver (Bian et al., 2010)	mmu-miR-202-5p, mmu-miR-122, mmu-miR-223, mmu-miR-134, mmu-miR-709, mmu-miR-720, mmu-miR-680, mmu-miR-494, mmu-miR-155
rat liver (Kren et al., 2009)	rno-miR-130a, rno-miR-130b, rno-miR-140*, rno-miR-320, rno-miR-494, rno-miR-671
myotubes (Barrey et al., 2011)	hsa-miR-720, hsa-miR-133b, hsa-miR-1974, hsa-miR-24, hsa-miR-133a, hsa-miR-125a-5p, hsa-miR-1979, hsa-miR-103, hsa-miR-125b, hsa-miR-103, hsa-miR-221, hsa-miR-23a, hsa-let-7b, hsa-miR-423-3p, hsa-miR-106a, hsa-miR-23b, hsa-miR-92a, hsa-miR-193b, hsa-miR-365, hsa-miR-93, hsa-miR-532-3p, hsa-miR-20a, hsa-miR-149, hsa-miR-181a, hsa-miR-503, hsa-miR-210, hsa-miR-107, hsa-miR-574-3p, hsa-miR-34a, hsa-let-7g, hsa-miRPlus-D1033, hsa-miR-19b, hsa-miR-197, hsa-miR-324-3p, hsa-miR-127-3p, hsa-miR-324-5p, hsa-miR-484, hsa-miR-151-5p, hsa-miR-486-5p, hsa-miR-542-5p, hsa-miR-199a-5p, hsa-miR-501-3p, hsa-miR-675*, hsa-miR-134, hsa-miR-490-3p, hsa-miR-598
HeLa (Sripada et al., 2012)	hsa-mir-1973, hsa-mir-1275, hsa-mir-494let-7b, let7g, hsa-miR-107, hsa-miR-181a, hsa-miR-221, hsa-miR-320a
143-B (Mercer et al., 2011)	hsa-mir-146a, hsa-mir-103, hsa-mir-16
HEK293 (Sripada et al., 2012)	let-7b, let7g, hsa-miR-107, hsa-miR-181a, hsa-miR-221, hsa-miR-320a

The validated miRNAs enriched/associated with mitochondria from different cell lines and tissues till date have been summarized.
[a] The cell or tissue from which mitochondria are isolated and reference.
[b] The validated list of miRNAs enriched/associated with mitochondria from specified source. The validation was done by either qPCR or Northern blotting.

membrane of mitochondria may provide novel platform to regulate the site specific mRNA and protein levels in the cell.

In conclusion miRNAs are both associated and localised in mitochondria. The presence of miRNAs and its RISC complex protein at mitochondria raises several questions. It is possible that the mitochondrial associated miRNAs regulate mitochondria encoded mRNA or nuclear encoded mRNA/protein destined to mitochondria. The mitochondria may be the vehicle to transport miRNAs and accessory protein to regulate the mRNA/protein level at a distant subcellular site. The further study in this direction will answer several interesting possibilities.

4.2. Unique and specific pattern of miRNA in mitochondria

The patterns of expression/association of proteins with mitochondria from different cells and tissue are remarkably different (Calvo and Mootha, 2010) depending on energy requirement, tissue size, diversity and complexity. Similarly, emerging evidences suggest that association of miRNAs is unique and cell type specific and may be again dependent upon metabolic demand of the cell and its origin. The study from our lab showed the unique association of sRNA with the mitochondria of HEK293 and HeLa. 21% and 27% of known miRNAs (as per miRBase 17.0) associated with mitochondria of HEK293 and HeLa respectively. The degree of association was diverse, ranging from 0.0014% to 14.08% (Sripada et al., 2012). The association of few miRNAs (let-7 family, hsa-miR-107, hsa-miR-103, hsa-miR-320 and hsa-miR-181) with mitochondria appears to be universal (Bandiera et al., 2011; Barrey et al., 2011; Sripada et al., 2012). The association of some miRNAs with mitochondria are cell type specific. For example 134 miRNAs including hsa-miR-128, hsa-miR-1307 and hsa-miR-140-3p associate significantly with mitochondria of HEK293 whereas 24 miRNAs including hsa-miR-23a, hsa-miR-181b and hsa-miR-30 associate significantly with mitochondria of HeLa (Sripada et al., 2012). The elucidation of universal/cell type specific enrichment and functionality of miRNAs in the mitochondria from cells of different origins may provide more insight to define the signature of mitochondrial specific miRNAs and their crosstalk with other organelles inside the cell.

4.3. Possible mechanisms of transport of miRNA to mitochondria

The processes of RNA import to the mitochondria has already observed in many organisms including protozoans, plants, fungi, yeasts and animals (Sieber et al., 2011). The A8344G and A3243G mutations in mitochondrial $tRNA^{Lys}$ and $tRNA^{Leu}$ causes MERRF (myoclonic epilepsy with ragged red fibers) and MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) syndrome respectively. The partial resumption of mitochondrial functions and syndromes were rescued by enhancing the import of required tRNA (Karicheva et al., 2011; Kolesnikova et al., 2004). There are numerous evidences describing mechanism of RNA import into mitochondria. The cytosolic tRNAGln is transported to mitochondria using ATP without any cytosolic factor or protein import system or by voltage dependent anion channel (VDAC) located in the outer membrane (Rubio et al., 2008).

The process of the miRNA import system is an interesting area to be elucidated. Recently, it was observed that polynucleotide phosphorylase (PNPASE), an inter membrane space (IMS) protein has a key role in import of small RNA. The enzyme has 3′–5′ exoribonuclease activity and degrades RNA trying to enter mitochondria. However, RNA with a stem loop structure and 0–4 nt overhang at 3′ end is inaccessible to catalytic domain thereby protecting from degradation (Wang et al., 2010). This may enhance specific structural ncRNA import into the organelle. PNPASE is conserved in bacteria, plants, flies, mice, and humans. In plants, it is localized both in the intermembrane space of mitochondria and chloroplast. *Arabidopsis* lacking chloroplast targeted PNPASE, cpPNPase (pnp 1–1) showed 98% difference in ncRNAs expression levels in chloroplast as compared to wild type (Hotto et al., 2011). The similar investigation of PNPASE in mitochondria may provide more clues of small ncRNA import.

Interestingly, Ago2 (co-localised with mitochondria) was found to be involved in the transport of a 70 nt tRNAMet from mitochondria to cytosol (Maniataki and Mourelatos, 2005). This suggests that components involved in miRISC assembly, especially Ago2 may be involved in transport of miRNAs across mitochondria. Therefore, it is important to study co localization of other proteins involved in miRNA biogenesis with mitochondria that may provide further clues of possible miRNA import. By contrast, it would be interesting to investigate the existence of a protein independent mechanism of miRNA import to mitochondria similar to RNA import complex (RIC) in Leishmania (Mukherjee et al., 2007). A detailed dissection of what constitutes a trigger sequence for processing versus import activities is clearly warranted.

4.4. Alignment of miRNA to mitochondrial genome

The semi-autonomous organelles: mitochondria and chloroplast originated by endosymbiosis of ancestral α-proteobacteria. These organelles have their own compact genome (devoid of introns) with high-copy numbers like their ancestors. The human mitochondrial genome is 16,569 bp circular dsDNA. It is known to encode bioenergetically important genes (13 proteins of ETC.), 22 tRNA and 2 rRNA. The replication and transcription of mitochondrial DNA (mtDNA) is initiated from the small non coding region, the D loop. It is also regulated by nuclear-encoded RNA and proteins (Anderson et al., 1981; Andersson et al., 2003). The recent studies demonstrating the presence of miRNAs in mitochondria suggests the possibility of regulation of mitochondrial encoded transcript. These miRNAs may fully or partially align to mitochondrial genome. 33 pre-miRNAs and 25 mature miRNAs aligned to mitochondrial genome from miRBase search engine (Barrey et al., 2011). 2 known miRNAs each from HEK293 and HeLa and 22 putative novel miRNAs from HEK293 aligned to mitochondrial DNA (Sripada et al., 2012). The origin of these miRNAs are either from mitochondrial or nuclear genome is not clear.

5. Targets of mitochondrial resident miRNA

The extensive validation of the targets of miRNAs associated with mitochondria has still been not studied. The bioinformatics analysis suggests that miRNAs associated with mitochondria targets both nuclear encoded and mitochondrial mRNA transcripts (Bian et al., 2010; Sripada et al., 2012). The experimental evidences suggest few nuclear encoded miRNAs translocates to mitochondria and regulates mitochondrial transcripts whereas the majority of miRNAs targets nuclear encoded mRNA. For example, hsa-miR-133a targets ND1 whereas hsa-mir-1, hsa-mir-181a/b and hsa-mir-206 (enriched in the muscle mitochondria) do not regulate transcripts of mtDNA (Barrey et al., 2011). Similarly, amongst 15 miRNAs identified in the rat liver mitochondria only one miRNA (hsa-miR-130) targeted mitochondrial gene (COX3) (Kren et al., 2009). These evidences suggest that mitochondrial genome encoded transcripts may not be the preferred targets of miRNAs associated with mitochondria (Barrey et al., 2011) but may be a transitory site for these important miRNAs to regulate the other targets.

The highly enriched miRNAs in muscle mitochondria like hsa-mir-31, hsa-mir-302a, hsa-mir-21 and hsa-mir-181b were previously reported to be upregulated in colorectal, thyroid carcinoma and myeloid leukaemia (Bandres et al., 2006; Chen et al., 2010; Iliopoulos et al., 2010; Tetzlaff et al., 2007). Similarly, it has been consistently observed in all the studies published so far including ours that tumour suppressor let-7 family members are enriched in mitochondria (Bandiera et al., 2011; Barrey et al., 2011; Sripada et al., 2012). The let-7 members are down regulated in several cancers and regulate both tumor invasion and apoptosis by targeting oncogenes (c-Myc, ras, high-mobility group A (HMGA), Janus protein tyrosine kinase (JAK), signal transducer and activator of transcription 3 (STAT3) and NIRF) (Wang et al., 2012b). p53 is also a putative target of hsa-mir-107, hsa-mir-145, hsa-mir-134, hsa-mir-503 and hsa-mir-21 which are detected in the mitochondria (Barrey et al., 2011). p53 in turn regulates some components of miRNA processing machinery (Drosha, Dicer, DGCR8, TARBP2, Exportin5) (Boominathan, 2010). The putative targets of miRNAs associated with mitochondria from HEK293 and HeLa are involved in RNA metabolism, transcription, protein metabolism, cell death, cell cycle and stress response (Sripada et al., 2012). These evidences suggest that mitochondria-associated miRNA may contribute actively in different pathological conditions.

6. Physiological implications of miRNA in mitochondria

The emerging evidences suggest that miRNAs may play an important role in developmental and pathological conditions. miRNA modulates the mRNA repression by binding to mRNA with its 5′ 2–8 nt sequence. The miRNA can target 100's of mRNA. Conversely many miRNAs may have similar seed sequence to target a single gene. The role of miRNA in regulation of mitochondrial is developing and important clues are emerging.

The miRNAs regulate nuclear encoded mitochondrial proteins at post transcriptional level consequently affect mitochondrial homeostasis, OXPHOS, ATP synthesis, apoptosis, ROS generation or fatty acid metabolism in several pathologies (Aroor et al., 2012). Similar findings have been summarized briefly in Table S2. The expression pattern of miRNAs in tissue as well as in mitochondria is altered in pathophysiological conditions. For example, the expression patterns of miRNAs in mouse liver and its mitochondria is altered in Streptozotocin (STZ) induced type 1 diabetes. The levels of mmu-miR-494, mmu-miR-202-5p, mmu-miR-134 and mmu-miR-155 significantly enriched in mitochondria in diabetic mice, whereas the levels of mmu-miR-705 and mmu-miR-122 remained unchanged (Bian et al., 2010). Recent study provided strong evidence of the translocation of hsa-miR-181c to mitochondria during myocardial infarction to regulate the mitochondrial complex IV and ROS levels (Das et al., 2012). Similarly, hsa-mir-133a regulates muscular activity during differentiation of myoblasts by targeting mitochondrial complex1 gene ND1 (Barrey et al., 2011). In our study, putative targets of mitochondria-associated miRNAs from HEK293 and HeLa included oncogenes, tumor suppressors, proapoptotic genes, apoptotic genes, antiapoptotic genes and cell cycle components. Analysis by KEGG, showed that mitochondrial associated miRNAs regulate important biological pathways: ubiquitin mediated proteolysis, TGF cycle, Wnt cycle, cell cycle, p53 and neurotrophin signalling (Sripada et al., 2012). This implies that mitochondria-associated miRNAs are involved in not only in regulation of mitochondrial functions but several other cellular processes.

7. Concluding remarks

Interestingly, the different components enclosed in the double membrane are more diverse as previously postulated and need more explorations. The recent observation of association of miRNA with/inside the mitochondria may have important implications in several cellular processes. The role of mitochondria clearly extends beyond its role in energy metabolism to apoptosis, inflammation and sensor of infections. The study in this direction will definitely decipher the role of mitochondria-associated miRNAs in the inter organellar cross talk and implications in various pathological conditions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mito.2012.10.009.

Acknowledgements

The work on mitochondria-associated miRNA in the lab (RS) is supported by Department of Biotechnology, Govt. of India (grant

number — BT/PR12906/AGR/36/638/2009). Dhanendra Tomar received Senior Research Fellowship from CSIR, Govt. of India. Lakshmi Sripada received Junior Research Fellowship from UGC, Govt. of India.

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