Chapter 3

Bax is the target of hsa-miR-128
miRNAs are a new class of non-protein-coding, small, highly conserved RNA molecules which are about 18–24 nt long. These tiny molecules play around the genes by regulating their expression either by translational repression or degradation of their target message (Pillai, 2005; Zamore and Haley, 2005). Complementarity between seed sequence (2-7 nt) of miRNA and 3'UTR of target mRNA will decide the fate of the target mRNA whether it will be cleaved or transcriptionally repressed by respective miRNA (Bartel, 2009). miRNAs are processed from longer transcripts in a sequential manner to stem-loop precursors in nucleus and finally to shorter ~22 nt mature miRNA in cytoplasm. It is estimated that miRNAs account for ~1% of the predicted genes in higher eukaryotic genomes and that up to 30% of genes might be regulated by miRNAs (Yu et al., 2007). They have been found to control a number of fundamental biological processes such as development, differentiation, cell proliferation, apoptosis, metabolism and stress responses in a variety of organisms ranging from Caenorhabditis elegans to plants and Drosophila melanogaster to mammals (including humans) (Chen et al., 2004; Cheng et al., 2005; Karp and Ambros, 2005; Lu et al., 2005). Their profound impact on these biological and patho-physiologic processes clearly shows that any aberration in miRNA biogenesis pathway or its activity contributes to several human diseases including cancer (Akkina and Becker, 2011; Dorn, 2011; Guay et al., 2011; Kerr et al., 2011; Lynam-Lennon et al., 2009).

Recent findings have demonstrated that miRNAs could act either as potential oncogenes or tumor suppressor genes (He et al., 2005; Volinia et al., 2006). At the time this work was initiated there were large number of discovered miRNAs (presently more than 1300 miRNAs are known in humans) and an amazingly large array of their bioinformatically predicted target genes but their regulatory mechanism and cellular functions were scarcely known. Keeping this in mind, the present study was designed to functionally characterize the miRNA(s) that regulate(s) apoptosis.
To begin with, we selected Bax gene and looked for the miRNAs which were predicted to target this gene. Bax is a cytosolic proapoptotic member of Bcl-2 superfamily. In healthy cells Bax remains 'dormant', located in the cytosol or loosely associated to the mitochondria. Upon induction of apoptosis, cytosolic p53 activates monomeric Bax by inducing its homo-oligomerization and the protein translocates to mitochondria. During translocation into the mitochondria, it gets inserted into the outer membrane, oligomerizes and triggers the pore formation and release of cytochrome c, Smac/Diablo and other proteins from the intermembrane space. These events require various conformational changes of the protein (Chipuk et al., 2004; Roucou and Martinou, 2001). Nuclear magnetic resonance spectroscopy (NMR) revealed the structure of Bax to consist of nine α helices. The overall fold of Bax closely resembles that of Bcl-xL (Muchmore et al., 1996) and Bid (Chou et al., 1999; McDonnell et al., 1999) with eight amphipathic α helices clustered around one central hydrophobic α helix (α5) (Suzuki et al., 2000). In its inactive cytosolic form, the two important domains of Bax-transmembrane domain (helix α9) and the BH3 domain, are masked inside the hydrophobic core of the protein (Chipuk et al., 2004; Roucou and Martinou, 2001). Such conformation would contribute to the solubility of the protein in the cytosol and prevent its membrane insertion in non-apoptotic cells.

From disease perspective, poor prognosis and resistance to chemotherapy in human ovarian and pancreatic cancers was found to be associated with reduced Bax mRNA and protein levels (Friess et al., 1998) (Tai et al., 1998). Loss of Bax expression was observed in endometrial carcinoma (Sakuragi et al., 2002) whereas high levels were found in colorectal cancer (Ogura et al., 1999). Additionally, Amyloid beta peptide upregulates Bax expression in human neurons during Alzheimer's disease (Paradis et al., 1996). Bax expression has been detected in Hodgkin and Reed-Sternberg (HRS) cells of Hodgkin’s disease (HD) (Rassidakis et al., 2002). The aberrant expression of
Bax during various disease conditions and little knowledge of regulation of Bax expression made it an interesting subject to study and we believe that finding miRNA(s) targeting this gene would add a new dimension to the ongoing research in the therapeutics.

3.2. Results

3.2.1. In-silico Analysis Reveals Bax as a Target of hsa-miR-128 and miR-22

It is widely accepted that miRNAs mediate gene regulation by reducing the stability of their target transcripts. Several target prediction softwares have been developed to predict the binding site of miRNA in the 3’UTR of their target mRNA. However, many false positive results are associated with them due to lack of specificity of prediction. To avoid the overprediction, we used a consensus approach employing 3 widely-used software, miRanda (John et al., 2004), RNAhybrid (Rehmsmeier et al., 2004) and Target Scan (Lewis et al., 2005), to perform the target prediction. Only those miR-target pairs predicted by all the three softwares were used in the analysis. We found that the transcript (NM_004324, ENST00000293288) of Bax harbored the hsa-miR-128 as well as hsa-miR-22 binding site in its 3’UTR. There was complete complementarity between the first nine nucleotides (including the 2- to 9-nt seed region) of hsa-miR-128 and 49–57 nt of 3’UTR of Bax (Fig. 3.1 A). However, there was partial complementarity between seed region of hsa-miR-22 and 3’UTR of Bax (3 nucleotides). miR-128 is encoded by two distinct genes, miR-128-1 and miR-128-2, which are processed into an identical mature sequence. MiR-128-1 and miR-128-2 are both intronic and are present on two different chromosomes. miR-128-1 is embedded in the R3HDM1 (R3H domain containing 1) gene on chromosome 2q21.3 and miR-128-2 is embedded in the ARPP21 (cyclic AMP-regulated phosphoprotein, 21 kDa) on chromosome 3p22.3, respectively. Because mature sequence is same for both genes, hereafter, we will be calling it miR-128 throughout the thesis.
Fig. 3.1: Prediction of miRNAs targeting pro-apoptotic gene Bax. A. Schematic representation of Bax and its 3’UTR indicating the binding site of hsa-miR-128 and miR-22 as predicted. First nine nucleotides of miR-128 and its target region (Bax 3’UTR): red colored, bold shows complete complementarity whereas first three nucleotides of miR-22 and its target region (Bax 3’UTR): red colored, bold shows sequence complementarity. Seed region is the sequence of first 2-9 nt of mature miRNA. B. Northern blot analysis was performed as described in materials and methods (chapter 2) for checking the basal expression of miR-128 and -22 in different cell lines. U6 was used as a loading control. Representative of three independent experiments is shown. C. Western blot analysis was performed as described in materials and methods (chapter 2) for Bax and β-actin in different cell lines. β-actin was used as a loading control. Representative of three independent experiments is shown.
3.2.2. Expression of hsa-miR-128, miR-22 and Bax Protein in Cell Lines

Before proceeding with the experiments, we checked for the expression of Bax protein by western blot analysis and hsa-miR-128 and miR-22 by northern blot analysis in different cell lines of different organ origin (NCI-H460, Hela, HEK293T, leukemic-Jurkat and glioma-U87MG cells) (Fig. 3.1 B-C).

As evidenced from Fig. 3.1 B-C, we found that hsa-miR-128 and Bax protein levels were very low in U87MG-glioma cells. However, the levels of miR-128 and Bax protein were inversely related in HEK293T and NCI-H460 cells as we observed high expression of Bax protein and moderate levels of hsa-miR-128 in HEK293T and NCI-H460 cells. Among these two cell lines, NCI-H460 cells were showing 75% transfection efficiency and HEK293T cells were showing 95% transfection efficiency. Therefore, we chose HEK293T cells for our further study. hsa-miR-22 expression was found to be high in NCI-H460, Hela and U87 cells. However, moderate levels were observed in HEK293T and Jurkat cells.

3.2.3. Cloning of hsa-miR-128 and 3'UTR of Bax

The sequence of hsa-miR-128 was retrieved from miRbase (http://microrna.sanger.ac.uk/sequences/), the 3'UTR sequence of Bax gene was retrieved from NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez/) and for primer designing, primer 3 software was used (http://frodo.wi.mit.edu/).

To construct a plasmid expressing miR-128, a 705-bp genomic sequence of ARPP21 gene spanning precursor miR-128 was amplified from human genomic DNA by PCR as described in materials and methods (Chapter 2). Amplified fragment was next cloned into pSilencer4.1 vector (Ambion, Austin, TX, USA) and designated as p(128). The method is briefly summarized in Fig. 3.2.

We next cloned full 3'UTR (165 nt) of Bax gene into pMIR-REPORT miRNA Expression Reporter Vector (Ambion, Austin, TX, USA) between SpeI and HindIII restriction sites using forward primer 5’-ACTAGTTGCGTTTTC CT TAGCTGTCT-3’ and reverse primer 5’-CCCAAGCTTAGCTAGGGT CAGAGGGTCATC-3’ as described in materials and methods (Chapter 2). The resulting plasmids were sequenced by TCGA (The Centre for Genomic Application, Delhi, India) to ensure accuracy. The method is briefly summarized in Fig. 3.3.
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Fig. 3.2: Strategy to clone hsa-miR-128. A. Schematic representation of cloning of miR-128 in pSilencer 4.1-CMV neo vector. The cloning was performed as described in materials and methods (Chapter 2) between the BamHI and Hind III restriction site. The PCR conditions are highlighted here. B. Result of sequencing is showing here. The sequencing was performed using M13 forward primer. The sequencing result of clone 3 was blast with hsa-miR-128-2 encoding gene sequence (ARPP21) for sequence similarity.
Fig. 3.3: Strategy of cloning of Bax. A. Schematic representation of cloning of Bax in pMIR-REPORT vector. The cloning was performed as described in materials and methods (Chapter 2) between the SpeI and Hind III restriction site. The PCR conditions are highlighted here. The colony PCR and sequencing result of Bax clones are shown here for confirmation. The sequencing was performed using M13 forward primer. The sequencing result of clone 6 was blast with Bax gene sequence for sequence similarity.
3.2.4. hsa-miR-128 Negatively Regulates Bax

To ascertain if hsa-miR-128 and miR-22 regulate Bax, we transfected HEK293T cells with hsa-miR-128 and miR-22 mimic in a dose dependent manner (p(128) dose=2µg and 4µg for hsa-miR-128 and miR-22 mimic dose=100nM and 200nM) and did northern blot and western blot analysis. We observed dose dependent increase in the expression of hsa-miR-128 (2.22- and 3-fold) and corresponding reduction in Bax protein expression (1.4- to 2.4-fold) following transfection of hsa-miR-128 (Fig. 3.4 A-B). However, no such change in the expression of Bax gene was observed with mimic miR-22 (Fig. 3.4 C). Hence, we further carried out our work with hsa-miR-128 only.

For further confirmation of regulation of Bax by hsa-miR-128, we performed similar experiments in NCI-H460 cells. Increased expression of mature form of hsa-miR-128 by 6.4- to 13.4-fold was determined by TaqMan based real-time PCR assay after overexpression of p(128) in a dose-dependent manner in NCI-H460 cells as compared to untransfected as well as non-specific control (Fig. 3.4 D). Similarly, there was 1.4- to 1.62-fold (p=0.032) decrease in the Bax protein levels in a dose-dependent manner in NCI-H460 cells after overexpression of p(128) (Fig. 3.4 E). To investigate whether the decrease of Bax protein levels was accompanied by a decrease of its mRNA levels, we measured the Bax mRNA levels by real-time RT-PCR analysis. As shown in Fig. 3.4 F, Bax mRNA levels were significantly decreased (~50%) after overexpression of miR-128 (Fig. 3.4 F).

Several reports in literature show that Bax translocates from cytosol to mitochondria in response to cell death stimuli (Chipuk et al., 2004; Roucou and Martinou, 2001). To confirm that the decrease in Bax expression was due to the downregulation by hsa-miR-128 and not because of translocation, henceforth we carried out subcellular fractionation to check the distribution of Bax protein in both cytosol and mitochondria by western blotting upon p(128) overexpression in HEK293T cells. As expected, we observed significant decrease in Bax levels in both cytosolic and mitochondrial fractions (Fig. 3.4 G) after p(128) overexpression. The above results suggest that hsa-miR-128 down regulates Bax.
Fig. 3.4: Negative regulation of Bax by hsa-miR-128. **A.** Northern blot analysis of total RNA extracted from untransfected HEK293T cells and HEK293T cells transfected with 2 or 4 µg p(128). Hybridization to the U6 small nuclear RNA is shown as a loading control. Graph shows relative hsa-miR-128 expression.*p<0.05 versus control. **B-C.** Western blot of Bax performed on total cell extracts from untransfected and transfected HEK293T cells. In **B.** cells transfected with 2 or 4 µg p(128) and in **C.** transfected with 100nM and 200nM mimic miR-22. β-actin was used a loading control. The protein band was quantified and normalized to β-actin. Graph is plotted as mean of three independent experiments. Error bars±SD, *p<0.05 versus control. Normalized ID values represent normalized integrated densitometric values. **D.** Taqman assay for mature miR-128 in NCI-H460 cells showing overexpression of miR-128 in cells transfected with 2 or 4 µg p(128). **E.** Western blot of Bax in NCI-H460 cells transfected with 2 or 4 µg p(128). Lower panel shows the graph plotted as mean of three independent experiments. Error bars±SD, *p<0.05 versus control. **F.** Real-time RT–PCR analysis of Bax expression in untransfected HEK293T and HEK293T cells transfected with 2 or 4 µg p(128). Data are expressed as the average±SD of three independent experiments performed in triplicate. *p<0.05 versus control. **G.** Subcellular fractionation of Bax protein in untransfected HEK293T and HEK293T cells transfected with either non-specific control or 2 or 4 µg p(128). Cytosolic and mitochondria extracts were prepared as described in materials and methods (Chapter 2). C represents Cytoplasmic fraction, M represents mitochondrial fraction. The purity of the fractions was determined by the expression of COX IV (mitochondrial specific protein). β-actin was used as a loading control. Blot is a typical representative of three experiment with similar results.
3.2.5. Bax is a Direct Target of hsa-miR-128 as indicated by Luciferase Assay

The bioinformatic search revealed that seed region of hsa-miR-128 binds to the 49–57 nt of 3'UTR of Bax. Hsa-miR-128 binding site at 49–57 nt of the Bax-3'UTR has been found to be highly conserved among two other species, i.e., *P. troglodytes* (Chimpanzee) and *M. mulata* (Rhesus) after comparing the human sequence for interspecies homology (as shown in Fig. 3.5 A). To verify whether Bax is a direct target of miR-128, a dual-luciferase reporter system was employed. We cloned the full 3'UTR of Bax (165 nt) into pMIR-REPORT miRNA Expression Reporter Vector (Ambion) as described in “cloning section” above. We transfected HEK293T and NCI-H460 cells with this Bax reporter construct in absence or in presence of p(128) or in combination with anti-miR-128 and measured the luciferase activity at 24 hours post transfection as described in “materials and methods” (Chapter 2). Luciferase activity was reduced by 50% in HEK293T cells and by 75% in NCI-H460 cells (Student’s t test, p<0.05) when Bax 3'UTR reporter vector was co-transfected with p(128) as compared to parental luciferase construct (without the Bax 3'UTR) (Fig. 3.5 B) and this suppression was relieved by anti-miR-128 treatment. Inhibition of hsa-miR-128 by anti-miR-128 led to increased firefly luciferase activity by 40% and 35% in HEK293T cells and by 20% and 30% in NCI-H460 cells at 30 and 60 nM concentrations of anti-miR-128, respectively. p(135a) (non-specific control) did not affect luciferase activity of Bax 3'UTR construct.

We next performed western blot analysis for Bax on cell lysates from HEK293T cells transfected with p(128), p(135a) (nonspecific control) and anti-miR-128 (100 nM) at 24 hours post-transfection. Quantitation of the Bax by western blot showed 1.2- to 1.8-fold (p=0.018) decrease in expression of Bax by miR-128 in a dose-dependent manner whereas addition of anti-miR-128 increased the Bax expression by 2.26-fold (p=0.02) (Fig. 3.5 C). However, non-specific control did not affect the Bax protein expression.
Fig. 3.5: hsa-miR-128 negatively regulates the Bax expression in HEK293T cells. A. Comparison of binding site of miR-128 in Bax 3'UTR in three different species. Target site of miR-128 in the 3'UTR of Bax is completely conserved in H. sapiens (Human), P. Troglodytes (Chimpanzee) and M. mulata (Rhesus). The miR-128 binding site has been mutated in Bax 3'UTR as shown by asterisks. B. Luciferase assay in HEK293T and NCI-H460 cells. Cells were cotransfected with pMIR-REPORT-Bax 3'UTR (wild type) with either p(128) or nonspecific control or in combination with p(128) + anti-miR-128 at 30 or 60 nM. Luminescence was measured at 24 hours post transfection. The luciferase activity relative to pMIR-REPORT (intact) was plotted. The bar diagram represents mean±SD for three independent experiments. *p<0.05 versus pMiR (Parental luciferase construct). #p<0.02 versus Bax 3'UTR + p(128). C. Upper panel shows western blot analysis for Bax after transfection of clone expressing miR-128 (p(128)) in a dose-dependent manner, either alone or with anti-miR-128 at 100 nM. NS control represents the non-specific control. The same blot was probed for β-actin for normalization. Data are representative of a typical experiment repeated three times with similar results. Lower panel shows the bar diagram represents mean±SD for three independent experiments. *p<0.05 versus untransfected. #p<0.02 versus 4 µg p(128). D. The reporter constructs including wild type or mutant Bax 3'UTR was cotransfected with either miR-128 or non-specific control. Relative firefly luciferase activities were normalized with the Renilla luciferase activities. The luciferase activity relative to non-specific control was plotted. The bar diagram represents mean±SD for three independent experiments. *p<0.05 versus control.
To demonstrate that miR-128 interacts with a specific target sequence localized in the Bax 3'UTR, an additional reporter mutant construct was generated in which the predicted miR-128 binding site cac tgtga in the 3'UTR of Bax mRNA was mutated with tgtgtga by site directed mutagenesis (site of mutation has been shown by asterisks in Fig. 3.5 A). The resulting mutant construct (mutant Bax 3'UTR) was cotransfected with non-specific control or p(128) in HEK293T cells. Similarly, wild-type Bax 3'UTR (wt Bax 3'UTR) was cotransfected with nonspecific control or p(128) in HEK293T cells and luciferase activity was measured. There was significant decrease (~50%) in luciferase activity when p(128) was cotransfected with the wild-type Bax 3'UTR as compared to when non-specific control (p(135a)) was cotransfected with Bax 3'UTR. However, there was no change in the luciferase activity when mutant Bax 3'UTR was cotransfected with p(135a) or p(128) (Fig. 3.5 D). These results strongly suggest that hsa-miR-128 directly inhibits the expression of Bax by binding to its target sequence.

### 3.3. Discussion

Several observations in our study suggest that hsa-miR-128 regulates Bax expression: for example, overexpression of miR-128 reduces both RNA as well as protein levels of Bax, whereas inhibition of miR-128 increases Bax expression in HEK293T and NCI-H460 cells. The ability of miR-128 to regulate Bax expression is likely to be direct as it binds to the 3'UTR region of Bax mRNA with complete complementarity to its seed region as shown by luciferase assay in both the cell lines. Our findings show that, in addition to transcriptional and post-transcriptional regulation, Bax can also be regulated at the translational level by miRNA.

Bax is a pro-apoptotic member of Bcl-2 superfamily, which is required for cell death and functions as a tumor suppressor. Absence of Bax expression has been observed in a number of tumors e.g. Breast tumor, lung cancer which confers growth advantages on cancer cells. Restoration of Bax expression in breast cancer cell lines inhibited tumorigenicity (Bargou et al., 1996) and
increased sensitivity to cytotoxic drug therapy (Wagener et al., 1996). Frameshift mutations occur in the BAX gene in colon and gastric cancers that lead to tumor progression (Yin et al., 1997). Bax also influences the prognosis of human pancreatic cancer (Friess et al., 1998) and it is a negative prognostic factor in non-small-cell lung cancer (Apolinario et al., 1997). Lewis et al. showed that Bax deficiency can promote rather than inhibit apoptosis in mice infected with Sindbis virus (Lewis et al., 1999). They also indicated that the anti-apoptotic versus pro-apoptotic function of Bax is determined by cell-specific factors. Zhang et al. in their study also showed that the absence of Bax completely abolished the apoptotic response to the chemopreventive agent sulindac and other nonsteroidal anti-inflammatory drugs (NSAIDs) in human colorectal cancer cells (Zhang et al., 2000). Wei et al. showed with knockout MEFs that Bak and Bax are functionally redundant and can substitute each other (Wei et al., 2001). Other studies also point towards the compensation of Bax by Bak in p53 null cells (Mondal et al., 2012) because Bak has faster killing kinetics than Bax i.e it gets activated faster than Bax by BH-3 only proteins including Puma (Kim et al., 2009).

Because of these conflicted roles of Bax in apoptotic process (as inducer or inhibitor of apoptosis), mechanisms regulating this important Bcl-2 family protein need to be elucidated further.