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Identification of targets of miR-200b by a SILAC-based quantitative proteomic approach

Arivusudar Marimuthu [a], *Tai-Chung Huang* [b], *Lakshmi Dhevi N. Selvan* [a,c], *Santosh Renuse* [a,c], *Raja Sekhar Nirujogi* [a,d], *Praveen Kumar* [a], *Sneha M. Pinto* [a,e], *Sudha Rajagopalan* [f], *Akhilesh Pandey* [b,g,h,i], *H.C. Harsha* [a,*], *Aditi Chatterjee* [a,*]

[a] Institute of Bioinformatics, International Technology Park, Bangalore 560066, Karnataka, India
[b] McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore 21205, MA, USA
[c] School of Biotechnology, Amrita University, Kollam 690525, Kerala, India
[d] Centre of Excellence in Bioinformatics, School of Life Sciences, Pondicherry University, Puducherry 605014, India
[e] Manipal University, Manipal 576104, Karnataka, India
[f] Agilent Technologies Pvt. Ltd., Bangalore 560048, Karnataka, India
[g] Department of Biological Chemistry, Johns Hopkins University, School of Medicine, Baltimore 21205, MD, USA
[h] Department Oncology, Johns Hopkins University, School of Medicine, Baltimore 21205, MD, USA
[i] Department Pathology, Johns Hopkins University, School of Medicine, Baltimore 21205, MD, USA

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ABSTRACT

miRNAs regulate gene expression by binding to cognate mRNAs causing mRNA degradation or translational repression. Mass spectrometry-based proteomic analysis is being widely used to identify miRNA targets. The miR-200b miRNA cluster is often overexpressed in multiple cancer types, but the identity of the targets remains elusive. Using SILAC-based analysis, we examined the effects of overexpression of a miR-200b mimic or a control miRNA in fibrosarcoma cells. We identified around 300 potential targets of miR-200b based on a change in the expression of protein levels. We validated a subset of potential targets at the transcript level using quantitative PCR.

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1. Introduction

MicroRNAs (miRNA) are 21–25 nucleotides long non-coding RNAs that pair with mRNAs to regulate gene expression. Genes that encode miRNAs are transcribed to produce long hairpin pri-miRNAs (primary transcripts). These pri-miRNAs are processed by drosha to produce pre-miRNAs, which are further processed to produce ∼22 nucleotide long mature miRNAs. The complementarity of seed sequence (six or seven nucleotides at 5′ end of mature miRNA) to the target mRNA determines the mode of gene regulation. Perfect

* Corresponding authors. Tel.: +91 8028416140; fax: +91 80 28416132.
E-mail addresses: harsha@ibioinformatics.org (H.C. Harsha), aditi@ibioinformatics.org (A. Chatterjee).

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complementarity results in degradation of target mRNA and imperfect complementarity leads to translational repression of the target transcript [1].

miRNAs are known to bind multiple targets and have been shown to regulate various biological process including development [2], differentiation [3], apoptosis and organogenesis [4,5]. Though thousands of miRNAs have been discovered, their function is yet to be clearly defined. In cancer, miRNAs have been shown to function both as oncogenes and tumor-suppressors [6]. miR-200b belongs to miR-200 family of miRNAs, which includes miR-200a, miR-200c, miR-429 and miR-141. The miR-200 family is implicated in negative regulation of epithelial to mesenchymal transition [7,8]. For example, miR-200b was found to be deregulated in neuroblastoma [9], serous ovarian carcinoma [10] and colorectal carcinoma [11]. Hu et al. have shown the prognostic value of miR-200b in ovarian cancers [12]. Aberrant expression of miR-200b is also known to enhance tumor growth [12]. Despite these studies the mechanisms by which miR-200b exerts its function remains unclear. Also, the number of identified miR-200b targets remain relatively limited thus precluding a complete understanding of the full oncogenic/tumor suppressor potential of this miRNA.

It is known that miRNA regulation at the translational level is more pronounced than the transcriptional level [13]. Hence this study is focused on identification of targets at the protein level using SILAC (stable isotope labeling by amino acids in cell culture)-based quantitative proteomic methodology [14]. SILAC is an unbiased method to identify differential protein expression. It exploits the use of different stable isotopic forms of amino acids, so that chemical nature of the medium across conditions remains the same except for subtle variations in the mass of stable isotopic amino acids used, which can be differentiated in the mass spectrometer. One of the major advantages of SILAC is that multiplexing occurs at the protein level reducing experimental errors in downstream processing. This method has been successfully used in previous studies to identify miRNA targets [15,16]. By this approach, we analyzed the expression profile of proteins in fibrosarcoma cells upon forced expression of miR-200b. Fibrosarcoma cell line HT-1080 is an excellent model to study the effects of miR-200b as this cell line exhibits high levels of ZEB1 which is a proven target of miR-200 cluster. Our results indicate that HT-1080 expresses low to undetectable levels of miR-200b, which is in agreement with previous report by Hurteau et al. [17].

Our results indicate that miR-200b regulates multiple proteins involved in tumor progression including cell cycle progression and cell proliferation. Most importantly, we identified 300 differentially expressed proteins as potential targets of miR-200b.

2. Materials and methods

2.1. Cell culture, miRNA treatment

Human fibrosarcoma cells (HT-1080) were grown in DMEM medium. The cells were either grown in regular DMEM media or SILAC media containing heavy isotopes of Arg ($^{13}C_6$) and Lys ($^{1}H_4$). The cells grown in normal media were mock transfected with 100 nm of miRIDIAN microRNA mimic, negative control (Dharmacon) and the cells grown in SILAC media were transfected with 100 nm miR-200b mimic (miRIDIAN hsa-miR-200b mimic, Dharmacon). The cells were lysed 48 h post-transfection in urea lysis buffer (9 M urea in 20 mM HEPES, pH 8). Protein estimation was carried out using Lowry's assay. The samples were pooled after normalization. Strong-cation exchange chromatography (SCX) was carried out as described earlier [18]. Briefly, dithiothreitol (5 mM) was used to reduce the pooled proteins followed by addition of 10 mM iodoacetamide resulting in alkylation. Protein digestion was carried out using Trypsin (modified sequencing grade; Promega, Madison, WI) for 16 h at 37°. Polysulfoethyl A column (PolyLC, Columbia, MD) (100 × 2.1 mm, 5 μm particles with 300 Å pores) was used for fractionation of digested peptides. Twenty five SCX fractions (0.5 mL) were collected from a 0–350 mM KCl gradient in the presence of 10 mM potassium phosphate buffer (pH 2.85), containing 25% acetonitrile for 70 min at a flow rate of 0.2 mL/min. Solvent A contained 10 mM potassium phosphate buffer, pH 2.85, 25% acetonitrile and solvent B contained 10 mM potassium phosphate buffer, 350 mM KCl, pH 2.85, 25% acetonitrile. The Desalting was done using C_{18}-stagetips. The cleaned fractions were dried and stored at −80 °C until mass spectrometry analysis.

2.2. LC–MS/MS analysis

The peptides from SCX fractions were reconstituted in 0.1% formic acid and analyzed using the 6520 Accurate-Mass Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) interfaced with nanoflow HPLC-Chip Cube system (Agilent Technologies, Santa Clara, CA, USA). The HPLC-Chip contains columns made up of a reversed-phase material Zorbax 300SB-C_{18}, particle size of 5 μm. The high capacity enrichment column (160 nl) was used whereas 15 cm analytical column was utilized for peptide separation. The samples were loaded on an trap column using Agilent 1200 series HPLC system at a flow rate of 4 μl/min using solvent A (0.1% formic acid). The peptides were eluted at the flow rate of 400 nl/min using a gradient of 3–40% Solvent B (90% acetonitrile in 0.1% formic acid). MS data was acquired using MassHunter Data Acquisition software (Version B.01.03, Agilent Technologies, Santa Clara, CA, USA). MS spectra were acquired in the range of *m*/*z* 350–1.800 followed by maximum of three MS/MS analyses with the scan range of *m*/*z* 50–2000. The duty cycle was set to 2.1 s with 1 MS scan per second followed by 3 MS/MS scans per second. The precursor selection was based on preference to charge state in the order of 2+, 3+ and >3+ ions and a second level preference to abundance. The mass spectrometer was operated at capillary voltage of 1950 V and fragmentor voltage of 175 V. Peptide isolation was set to medium (4 *m*/*z*) and collision energy slope of 3 V plus offset of 2 V.

2.3. Data analysis

The raw mass spectrometry data was searched using Spectrum Mill proteomics workbench software (Rev. A.03.03, Agilent Technologies). The raw data was searched against Human RefSeq database (Build 47; 34,906 sequences) using Spectrum Mill algorithm. Trypsin was selected as protease

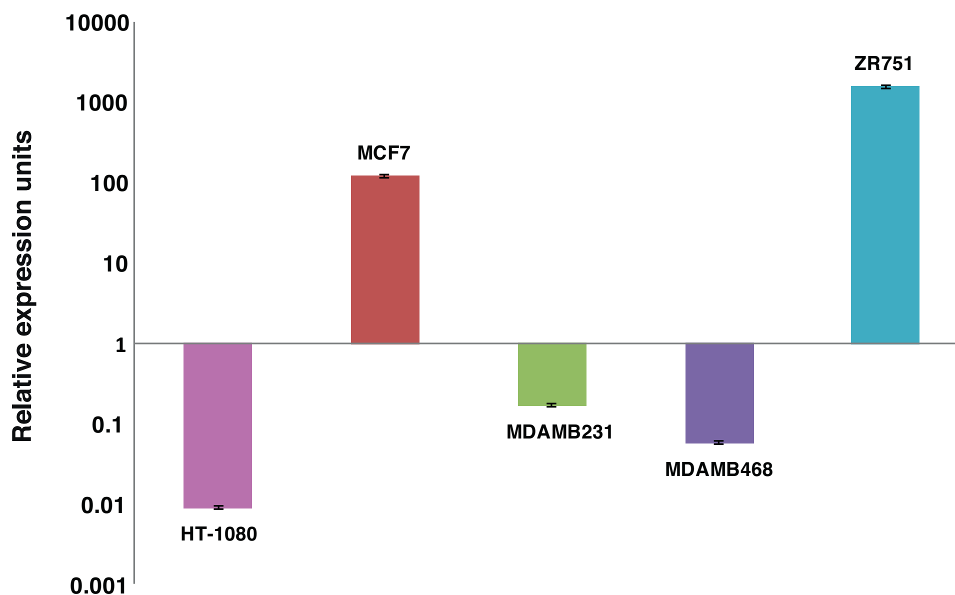

Fig. 1 – Endogenous expression of miR-200b in HT-1080. Endogenous expression of miR-200b in HT-1080 as compared to other cell lines as determined by quantitative PCR (TaqMan MicroRNA assay for miR-200b). Among the cell lines tested, HT-1080 shows the lowest expression of miR-200b.

with maximum one missed cleavage allowed. Oxidation of methionine, $^{13}C_6$ arginine and D4 lysine were selected as variable modifications while carbamidomethylation of cysteine was selected as a fixed modification. MS tolerance was set to 20 ppm while MS/MS tolerance was set to 0.8 Da. False discovery rate (FDR) was estimated based on decoy database searches. Applying FDR cut-off of 1%, 3751 unique peptide spectral matches were obtained corresponding to 1975 non-redundant peptides. The peptides were then grouped to proteins using default parameters provided in the Spectrum Mill search engine (protein score > 11, % SPI > 60). The "DEQ ratios" field was checked in the Spectrum Mill to compute relative quantitation of proteins.

2.4. *Experimental validation of the identified targets*

2.4.1. *qPCR validation*

Endogenous expression of miR-200b in different cell lines was determined by performing real time PCR using TaqMan® MicroRNA Reverse Transcription Kit, TaqMan Universal PCR Master Mix and TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA). CT value of miR-200b was normalized to the CT value of U6B (a small RNA) in the same sample. The expression levels of potential miR-200b target genes were measured using SYBR Green PCR System (TaqMan) as previously described [13]. Briefly, total RNA was extracted from the transfected cells using miRNeasy kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using Quantiscript Reverse Transcription Kit (Qiagen). For qRT-PCR, 1 μl of gene primers with SYBR Green (Applied Biosystems) in 20 μl of reaction volume was applied and analyzed using Rotor-Gene Q Real time cycler (Qiagen). The primers used in this study are provided in Supplementary Table 3. Target gene mRNA levels were normalized to UTP14A (control) mRNA according to the following formula: [2 − (CT target − CT UTP14A)] × 100%, where CT is the threshold cycle. The relative expression was calculated by dividing the normalized target gene expression of the miR-200b transfected sample with that of the mock transfected.

Supplementary Table 3 related to this article can be found, in the online version, at doi:10.1016/j.euprot.2014.04.006.

2.5. *Data submission*

We have deposited the raw mass spectrometry data (.d files) to proteomics data repository – Tranche (https://proteomecommons.org/tranche/). These files can be visualized using Agilent MassHunter Qualitative Analysis software (Agilent Technologies). The data can be downloaded using following hash:

6H4E7I0l66VE9lOUdhMXPTxKvhTFVsL64sJcfC+Xoz//
OTtebZXKVySQ9byKu+8T3sutjo0yDd2TpjFBVm2k2VmXdU
0AAAAAAAIj9Q==.

3. Results

In order to identify targets of miR-200b, we employed a quantitative proteomic approach using HT-1080, a fibrosarcoma cell line expressing low to undetectable levels of miR-200b (Fig. 1). The workflow employed in this study has been shown in Fig. 2. HT-1080 cells were either transfected with miR-200b mimic or the control miRNA. The HT-1080 cells expressing exogenous miR-200b were grown in DMEM media with heavy arginine and lysine, the cells harboring the control miRNA were grown and maintained in normal media. Forty eight hours post transfection the cells were harvested. Normalized protein amounts from the two states were mixed, followed

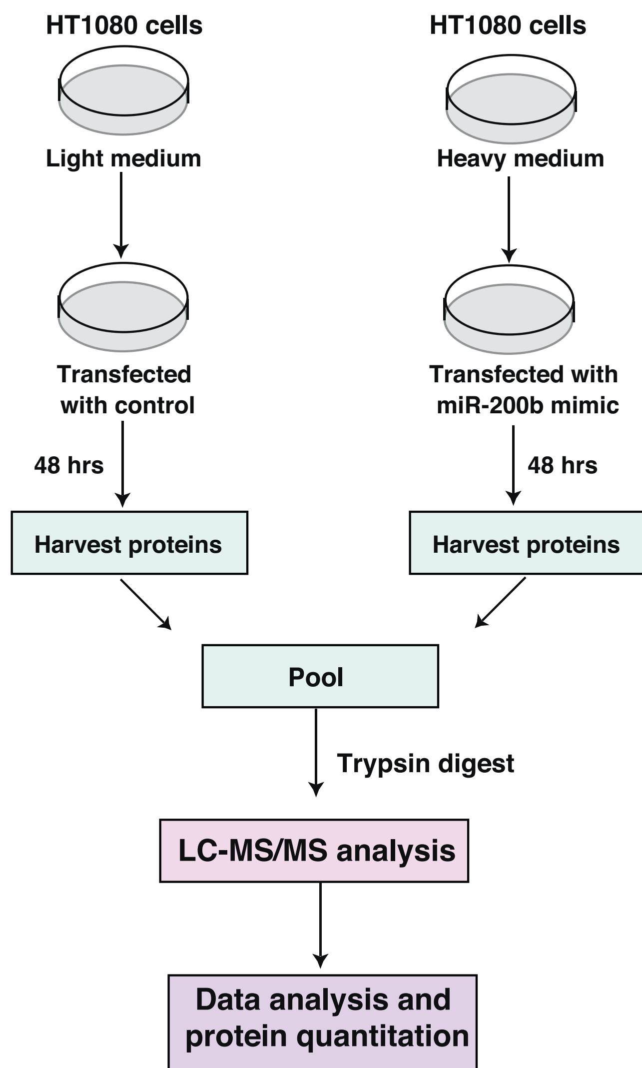

Fig. 2 – Workflow employed in this study: HT-1080 cells were either grown in normal media or adapted in SILAC media supplemented with heavy arginine($^{13}C_6$) and lysine (D4). The cells grown in normal media were mock transfected and the cells grown in SILAC media were transfected with miR-200b mimic. Forty eight hours post transfection the cells were lysed in urea buffer. The samples were pooled after normalization and subjected to trypsin digestion followed by fractionation using SCX chromatography. Fractionated samples were subjected to LC–MS/MS analysis on a Q-TOF mass spectrometer. The raw data was searched and quantitated using Spectrum Mill proteomics workbench.

by in-solution digestion and SCX fractionation, followed by LC/MS–MS analysis.

3.1. *LC–MS/MS analysis*

LC–MS/MS analysis of HT-1080 cells overexpressing miR-200b compared to the parental cell line led to the identification of 999 proteins. The proteins were identified by searching the spectra against human RefSeq protein database. Differential expression of proteins in miR-200b transfected vs control transfected cells were quantified using Spectrum Mill. Proteins with a ratio of ≥2 were considered as upregulated and with a ratio of ≤0.5 were considered as downregulated. Previous studies from our group as well as other miRNA research groups have shown that global effects of miRNAs on protein expression levels are often moderate and subtle [13,19–21]. Aforementioned studies have reported that the average change observed at protein level was between 10 and 35%. Based on these observations, a fold-change of 2 was selected to be sufficiently stringent to preclude false positives even with the known risk of losing some real candidates as false negatives. With this criterion, 300 proteins were found to be differentially expressed with majority of the proteins getting downregulated. The complete list of identified proteins and peptides are provided in Supplementary Tables 1 and 2, respectively.

Supplementary Tables 1 and 2 related to this article can be found, in the online version, at doi:10.1016/j.euprot.2014.04.006.

3.2. *Bioinformatics analysis*

The potential targets of miR200b identified above were then compared with the known predicted targets of miR-200b using TargetScan (http://www.targetscan.org) and miRDB (http://www.miRdb.org). From the potential targets identified from our study, 60 were predicted targets of miR-200b either by TargetScan or miRDB, 19 proteins were predicted targets by both. In addition to the above, we identified more than 200 novel targets of miR-200b which include ANXA1 (Annexin A1), LGALS3 (Galectin 3), HMGB1 (High mobility group protein 1) and EIF5A (Eukaryotic translation initiation factor 5A-1). MS/MS spectra of a subset of these candidates are shown in Fig. 3 (Table 1).

3.3. *Real-time PCR analysis*

miRNA regulates by binding to its cognate mRNA followed by transcriptional and/or translational repression. Next we validated the miR200b novel targets identified in this study at the transcript level using quantitative real-time PCR. Thirteen of the novel potential targets including, *CAST* (Calpastatin), *LGALS3* (Galectin-3), *ANXA1* (Annexin A1), *HMGB1* (High mobility group protein B1), *FN1* (Fibronectin 1), *CDK6* (Cyclin-dependent kinase 6), *EIF5A* (Eukaryotic translation initiation factor 5A), *PAK2* (p21activated protein kinase 2), *TRAP1* (TNF-receptor associated protein 1), *PRDX1* (Peroxiredoxin-1), *PDCD5* (Programmed cell death protein 5), *HINT1* (Histidine triad nucleotide-binding protein 1), *CSE1L* Exportin-2, (downregulated) were analyzed (Fig. 4). As depicted in the figure, the blue bars represent expression of the genes at the transcript level in cells transfected with control mimic whereas the red bar represents the transcript levels in cells transfected with miR-200b. Our data indicates that except for CSE1L, HMGB1 and PDCD5, the other potential targets show transcriptional downregulation in presence of miR-200b. Of the 13 tested candidates, eight were in concordance with the proteomic data (downregulated ≥1.6 fold at the mRNA level) (Supplementary Table 4).

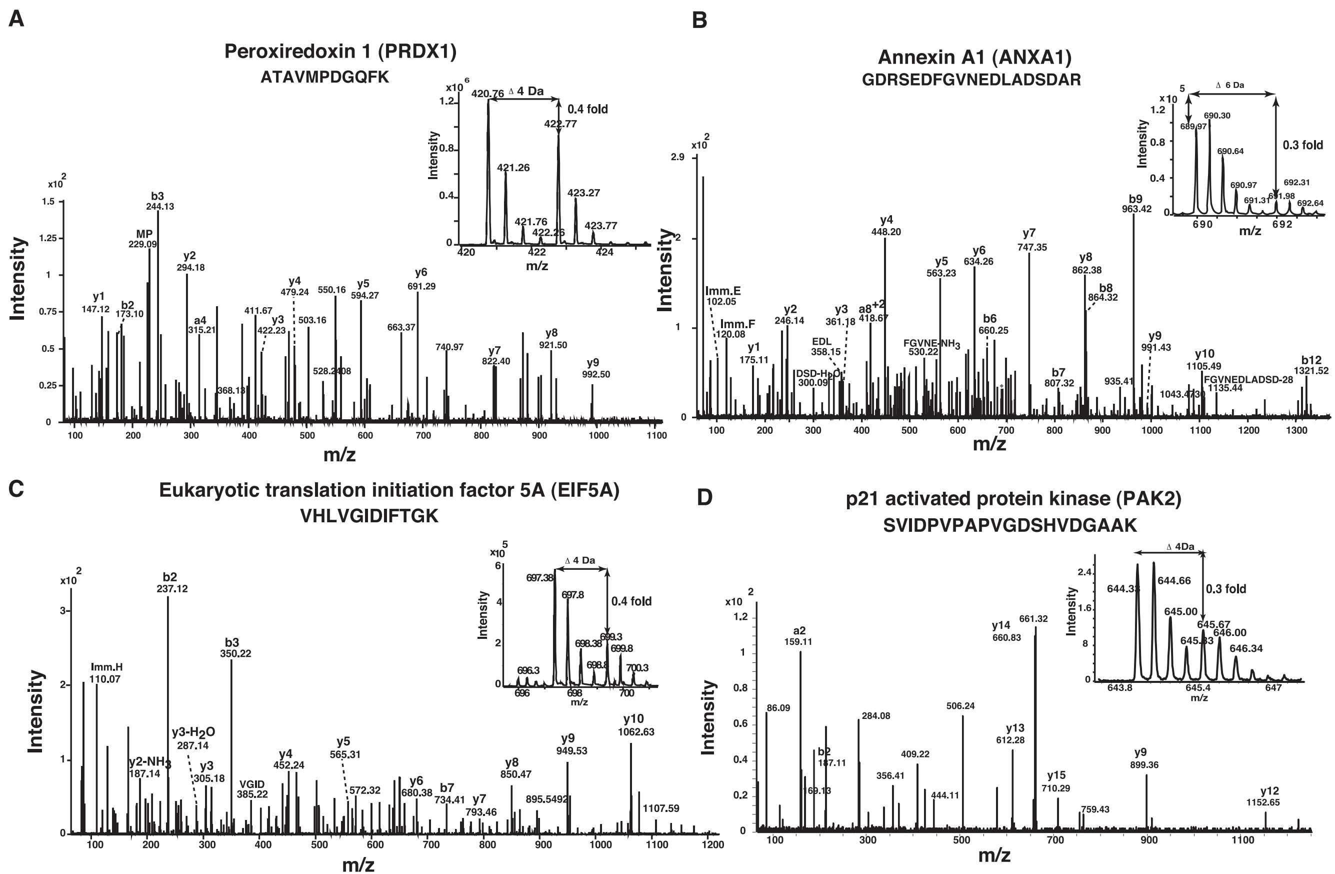

Fig. 3 – MS and MS/MS spectra of representative peptides of proteins. (a) Peroxiredoxin 1 (PRDX1), (b) Annexin A1 (ANXA1), (c) Eukaryotic translation initiation factor 5A (EIF5A), and (d) p21activated protein kinase 2 (PAK2).

Supplementary Table 4 related to this article can be found, in the online version, at doi:10.1016/j.euprot.2014.04.006.

4. Discussion

Both computational prediction and experimental approaches are commonly used to determine and predict miRNA targets. Computational algorithms such as TargetScan, miRanda, Target boost, Pictar and PITA are broadly used to predict targets [22]. Experimental identification of targets is largely driven by transcriptomic approaches including DNA microarrays, quantitative real time PCR-based assays; pull down assays for argonaute complexes. One of the major drawbacks of the above mentioned methods is that they are unable to readout the effect of miRNA on its target protein. Mass spectrometry-based proteomics approach have been used successfully to elucidate miRNA targets [22]. We and others have successfully used this platform for identification of miRNA targets

Table 1 – A subset of novel candidate targets of miR-200b identified in this study.

Gene symbol	Protein name	Fold downregulation
CAST	Calpastatin	12.5
LGALS3	Galectin-3	10.0
ANXA1	Annexin A1	3.8
HMGB1	High mobility group protein B1	3.4
FN1	Fibronectin 1	2.9
CDK6	Cyclin-dependent kinase 6	2.8
EIF5A	Eukaryotic translation initiation factor 5A-1	2.7
PAK2	Serine/threonine-protein kinase PAK 2	2.6
DNM1L	Dynamin-1-like protein	2.5
TRAP1	TNF-receptor associated protein 1	2.3
PRDX1	Peroxiredoxin-1	2.3
PDCD5	Programmed cell death protein 5	2.3
HINT1	Histidine triad nucleotide-binding protein 1	2.2
CSE1L	Exportin-2	2.0

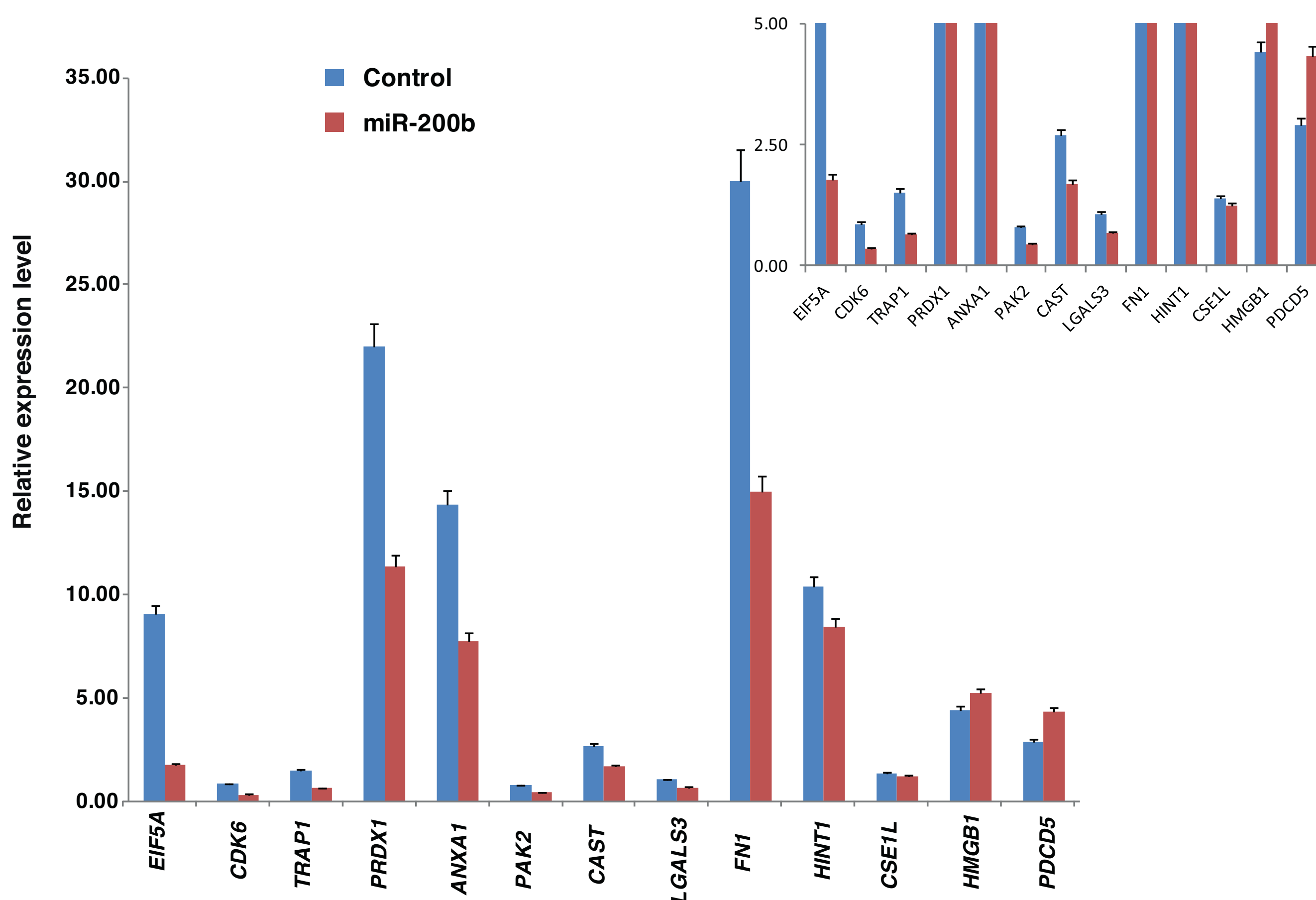

Fig. 4 – Verification of a subset of the identified targets by qPCR analysis. Relative expression levels of a subset of target mRNAs in mock transfected and miR-200b transfected HT-1080 cells are shown. The inset shows the expression of genes at a magnified scale to facilitate better visualization of the expression of *CDK6*, *TRAP1*, *PAK2*, *CAST* and *LGALS3*.

[13,15,19,23]. With the advent of quantitative proteomics, miRNA targets that undergo translational repression can be successfully identified which would be otherwise overlooked in experimental approaches that measures mRNA. Different labeling strategies including SILAC [15], iTRAQ [13], ICAT [24] – based proteomic approaches have been employed to discover targets of miRNAs.

The cluster of miR-200 miRNAs have been reported to play a major in role in multiple cancer types [12]. Although the tumor suppressor nature of miR-200 family of miRNAs have been established [25–27], the underlying targets and signaling cascade that are dysregulated remain undefined. miR-200b has also been shown to play a protective role in cancer progression through negative regulation of epithelial to mesenchymal transition [6]. Using LC–MS/MS analysis, we have identified more than 200 potential targets of miR-200b which were >2-fold downregulated. Many potential targets of miR-200b have known association with cancer progression. For example, overexpression of protein tyrosine phosphatase 11 (PTPN11), a tyrosine phosphatase with SH2 domain, has been shown to be involved in breast cancer progression [28]. Similarly, overexpression of 14-3-3 gamma (YWHAG), has been shown to promote invasion and metastasis in hepatocellular carcinoma [29]. Mucosae associated lymphoid translocation gene 1 (MALT1) is a known oncogene that is rearranged in chromosomal translocation with the apoptosis inhibitor gene *API2* [30] or with microtubule-associated protein 4 *MAP4* [31]. *MALT-API2* gene fusion has been reported as commonly occurring translocation in mucosa associated lymphoid tissue lymphomas [32]. These proteins were identified as targets of miR-200b in our study where forced overexpression of miR-200b led to a decrease in expression of the above mentioned proteins PTPN11, YWHAG and MALT1 by 2-fold, 2-fold and 4-fold, respectively. Another well established oncogene whose role has been implicated in multiple cancers is eukaryotic translation initiation factor 5A (EIF5A). Overexpression of this protein has been associated with poor prognosis in hepatocellular carcinoma [33]. It is the only known protein in eukaryotes which carries the unusual aminoacid hypusine. BCR-ABL positive cells treated with hypusination inhibitors (EIF5A inhibitor) has shown antiproliferative effects in K562 cells [34] confirming the oncogenic activity of EIF5A. Our study indicates EIF5A as a potential target of miR-200b as it was 4-fold downregulated in presence of overexpression of miR-200b. Moesin is another known oncogene, reported in HNSCC, and a target of miR-133a, which too is known to have a tumor suppressive role by targeting the moesin (*MSN*) gene [35]. In this study we identified moesin as a novel target of miR200b. Mosein was

observed to be downregulated by 2-fold in the presence of miR-200b.

Apart from being a tumor suppressor, miR-200b also functions as an oncomiR in certain cancers [36,37]. In the current study, we could identify miR-200b target proteins which were significantly downregulated and has literature support of having tumor suppressor role in multiple cancer types. Calpastatin (CAST), showed 12-fold downregulation by miR-200b. Low expression of CAST has been shown to be associated with lymphovascular invasion in breast cancer [38]. High mobility group protein B1 (HMGB1) has been reported to maintain DNA integrity by regulating DNA repair [39,40]. It has been reported to activate apoptotic response in case of genotoxic stress [40]. Depletion of this protein has been shown to promote carcinogenesis. In accordance with these observations we found a 3-fold downregulation of HMGB1 in response to exogenous expression of miR-200b in HT-1080 cells. These studies suggest that miR-200b might be involved in tumor progression. Further, evidence in support of the oncogenic role of miR-200b comes from the involvement of programmed cell death 5 (PDCD5) in tumorigenesis, which is downregulated in our study. Low expression of this protein in patients with chondrosarcoma had poor prognosis as compared to patients with high expression. Other proteins which were downregulated in response to exogenous expression of miR-200b in HT-1080 were HINT1 (2-fold), ANXA1 (4-fold) and LGALS3 (10-fold). Decreased expression of these proteins has been shown to be involved in tumor progression in different cancer types. Histidine triad (HIT) family of proteins are small family of nucleotide binding proteins whose expression is shown to be lost in multiple human malignancies [41]. In hepatocellular carcinoma, histidine triad nucleotide binding protein 2 (HINT2), a nuclear encoded mitochondrial protein has been reported to be downregulated. Restoration of the expression of this protein has been shown to induce apoptotic response in HEPG2 cells [42]. Loss of annexin A1 expression has been shown to inversely correlate with the progression of oral squamous cell carcinoma. Poorly differentiated carcinoma cells have been shown to have significantly lower expression of this protein as compared to well differentiated cells [43]. Downregulation of Galectin-3 (LGALS3) has been reported in tumor progression in cervical and prostate carcinomas [44,45]. These studies further support the notion that miR-200b behaves as an oncogene and plays a role in tumor progression.

In the present study using mass spectrometry-based approach, we have identified potential targets of miRNA. Further experiments are warranted to understand whether these are direct or indirect target of miR-200b. This study provides a lead for further research on identified potential targets. In summary, we have identified 300 proteins as candidate targets of miR-200b using a global quantitative proteomic approach. Eight out of 13 tested targets were confirmed to be regulated at transcription level. Forced expression of miR-200b has led to dysregulation of its targets. A handful of these targets have been identified as oncogenes in other studies, implicating miR-200b to possess a tumor suppressor role, whereby in the current study these targets were observed to be downregulated. Also, studies have indicated miR-200b to possess oncogenic property. In concordance with such observations, we too identified downregulated targets of miR-200b, whose loss or decrease in expression has been associated with cancer progression. In depth studies are required to elucidate the combined role of the identified targets to further confirm the role of miR-200b in cancer progression.

Conflict of interest

Sudha Rajgopalan is an employee of Agilent Technologies Pvt. Ltd. All other co-authors declare no conflict of interest.

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