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Heat shock factor 1 regulates hsa-miR-432 expression in human cervical cancer cell line

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ABSTRACT

Heat shock response pathway is a conserved defense mechanism of mammalian cells to maintain protein homeostasis against proteotoxic environmental conditions. This is characterized by robust synthesis of molecular chaperones mostly by stress-induced activation of heat shock factor 1 (HSF1). MicroRNAs (miRNAs) are a family of small non-coding RNAs that negatively regulate expression of protein-coding genes. Here we report altered expression of a set of miRNAs by thermal stress in HeLa cells. We also show that HSF1 regulates hsa-miR-432 expression in heat shock-dependent manner through its cognate binding site present in hsa-miR-432 upstream sequence. Our report uncovers a novel function of HSF1 and indicates involvement of miRNAs in HSF1-mediated protection of cellular proteome.

1. Introduction

The heat shock response (HSR) is an evolutionary conserved defense mechanism of cells for protecting themselves against acute proteotoxic stress, initiating a regulatory cascade for recovery and adaptation [1]. The main regulator of heat shock response is the transcription factor heat shock factor 1 (HSF1), which induces transcription of genes encoding heat shock proteins (HSPs). HSPs function as molecular chaperones aiding the folding of misfolded proteins and preventing protein aggregation [2,3]. In unstressed cells, inactive HSF1 monomers exist as part of multichaperone inhibitory complex. Upon stress, HSF1 is released from the complex, undergoes trimerization, post-translational modifications and nuclear accumulation, thus becomes transcriptionally competent and regulates transcription of its target genes by binding to specific DNA sequence called heat shock elements (HSEs) [4]. Apart from protection of cells from proteotoxic stress, HSF1 also regulates protein degradation, vesicular transport, cytoskeleton formation etc. via its ability to regulate expression of myriad of genes [5–7].

MicroRNAs (miRNAs) are small non-coding RNAs that act as negative regulators of gene expression with profound impact on a wide array of biological processes [8–10]. Besides, miRNAs are also involved in different diseases including neurodegenerative disorders, cancer, cardiovascular disease etc. [11,12].

Altered expression of miRNAs by thermal stress has been reported earlier [13,14], however the molecular mechanism behind such alteration is yet unknown. Here we identify a set of miRNAs whose expression is altered by heat shock in HeLa cells. Moreover we showed that HSF1 can regulate hsa-miR-432 expression through a functional HSE present in upstream sequence of the miRNA. We for the first time report that HSF1 can regulate miRNA expression and hsa-miR-432 is a novel transcriptional target of HSF1.

2. Materials and methods

2.1. Antibody and chemicals

HSF1 antibody was purchased from Abcam. TRIzol and Lipofectamine 2000 were obtained from Invitrogen. Other chemicals were procured locally.

2.2. Cell culture and treatments

HeLa cells were obtained from National Cell Science Centre, India and grown in Minimal Essential Medium (Himedia) supplemented with 10% fetal bovine serum (Biowest) at 37 °C in 5% CO_2

Abbreviations: ChIP, chromatin immunoprecipitation; HS, heat shock; HSE, heat shock element; HSF1, heat shock factor 1; HSP, heat shock protein; HSR, heat shock response; miRNA, microRNA; qRT-PCR, quantitative real time PCR; sqRT-PCR, semiquantitative RT-PCR.

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atmosphere under humified conditions. To induce heat shock response, cells were subjected to heat shock at 42 °C for 60 min and kept at 37 °C for 4 h. Transfection of cells was performed using Lipofectamine 2000.

2.3. Construction of plasmids

Human *hsf1* gene cloned in pcDNA3.1 vector was provided by Dr. Richard Voellmy (HSF Pharmaceuticals, Switzerland). Empty pSUPER vector and pSUPER constructs for HSF1 siRNA and scrambled RNA were gifted by Dr. L Sistonen (Åbo Akademi University, Finland).

For luciferase reporter assay, upstream sequence of hsa-miR-432 encompassing putative HSE (designated as miR-432_ups) was cloned in pGL3 vector (Promega) between the restriction sites of MluI and HindIII. Promoter sequence of human *hsp70* gene containing previously validated HSE (designated as Hsp70_ups) was used as positive control as described previously [15]. Primers used for cloning:

F: 5′-CGACGCGTACTCAAACACTTCGGACATGG-3′
R: 5′-CCCAAGCTTCAAAGAGCAACAGAGAGTAGCA-3′

2.4. Luciferase assay

Method used for luciferase assay was previously described [15]. HeLa cells expressing empty pGL3 vector or miR-432_ups or Hsp70_ups together with empty pcDNA vector or HSF1-pcDNA were either grown at 37 °C (control) or subjected to heat shock at 42 °C for 60 min followed by recovery at 37 °C for 4 h. Twenty-four hours after transfection, luciferase assay was carried out using luciferase reporter assay system (Promega) according to manufacturer's protocol and detected by Sirius Luminometer (Berthold Detection Systems). The experiments were carried out in triplicates.

2.5. RNA preparation

Total RNA was prepared from cultured cells using TriZol Reagent following manufacturer's protocol and was quantified using Biophotometer (Eppendorf).

2.6. Quantitative real-time PCR (qRT-PCR)

Total RNA (100 ng) was used for cDNA preparation using Taqman miRNA-specific stem-loop primers (Applied Biosystems) or stem loop primers designed as described in [16] along with MuLv-Reverse transcriptase (Fermentas), RNase inhibitor and dNTPs. cDNA was then subjected to either Taqman qRT-PCR (Applied Biosystems) which detects only mature miRNAs [17] or qRT-PCR using SYBR Green (Applied Biosystems) with miRNA-specific forward primer and universal reverse primer as described previously [18] using 7500 real time PCR system (Applied Biosystems). Fold changes were calculated in accordance with SDS software V2.0. Expression of hsa-miR-17-5p (which was unaltered by heat shock treatment) and *β-actin* were taken as endogenous control. The sequences of miRNA stem loop specific primers for cDNA preparation, miRNA specific forward primers and universal reverse primer for qRT-PCR using SYBR Green are given in Supplementary Table S1. Expression of *β-actin* was measured by following primers:

F: 5′-TCCTGTGGCATCCACGAAACT-3′
R: 5′-GAAGCATTTGCGGTGGACGAT-3′

2.7. Chromatin Immunoprecipitation

Methods used for ChIP were earlier described [15]. Immunoprecipitation was done using anti-HSF1 antibody (ab2923, Abcam). PCR amplification of input and immunoprecipitated DNA was carried out using primers flanking the putative HSF1-binding site located upstream of hsa-miR-432. Primers used for ChIP assay are:

F: 5′-ACTCAAACACTTCGGACATGG-3′
R: 5′-CAAAGAGCAACAGAGAGTAGCA-3′

2.8. Knockdown of HSF1 by siRNA

Method used for siRNA-mediated knockdown of endogenous HSF1 was as described in [19]. Briefly, HeLa cells were transfected with empty pSUPER vector or HSF1-specific siRNA or scrambled RNA cloned in the same vector. Cells were harvested after 72 h of transfection. Knockdown of endogenous HSF1 in HeLa cells was earlier confirmed by sqRT-PCR and Western blot [15].

2.9. Enrichment analysis

Validated targets of thermally altered miRNAs were collated from miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/). Enrichment of different GO biological processes, GO molecular functions and KEGG pathways was performed by GeneCodis (http://genecodis.cnb.csic.es/analysis/).

2.10. Statistical analysis

For statistical analysis, unpaired t test was done to compare the means of two experimental groups using the software GraphPad QuickCalcs.

3. Results

3.1. Heat shock alters miRNA expression in HeLa cells

In an attempt to identify the miRNAs whose expression are altered by heat shock, HeLa cells were either grown at 37 °C (control) or exposed to heat shock (HS) treatment at 42 °C for 1 h followed by recovery at 37 °C for 4 h. RNA was prepared from unstressed (control) and stressed cells and expression of 38 mature miRNAs was measured by quantitative real time PCR (qRT-PCR). Out of the 38 miRNAs tested, expression of 8 miRNAs was altered in stressed cells compared to unstressed cells (Fig. 1, Supplementary Table S2). miRNAs showing more than 1.5-fold change (increase or decrease) in expression ($p < 0.05$) by heat shock was considered as 'thermally altered' miRNAs. By this criteria, 7 miRNAs namely hsa-miR-34a, hsa-miR-125b, hsa-miR-128b, hsa-miR-146a, hsa-miR-205, hsa-miR-221 and hsa-miR-432 were identified whose expression was significantly upregulated by HS treatment in HeLa cells. Expression of one miRNA, hsa-miR-154 was significantly downregulated by the same treatment. As evident in Supplementary Table S2, expression of hsa-miR-214 was upregulated by more than 1.5-fold in response to heat shock however the change was not statistically significant. On the other hand, hsa-miR-148a expression increased significantly by HS treatment but the fold change was below the threshold value. Therefore hsa-miR-214 and hsa-miR-148a were not considered as thermally altered miRNAs.

3.2. Identification of heat shock elements (HSEs) in upstream sequence of thermally altered miRNAs

To address the question whether thermally altered miRNAs could be regulated by HSF1, we searched upstream sequence of all thermally altered miRNAs for presence of any putative HSE using the in-house tool described earlier [20]. Since the length and exact genomic location of most of the primary miRNAs are

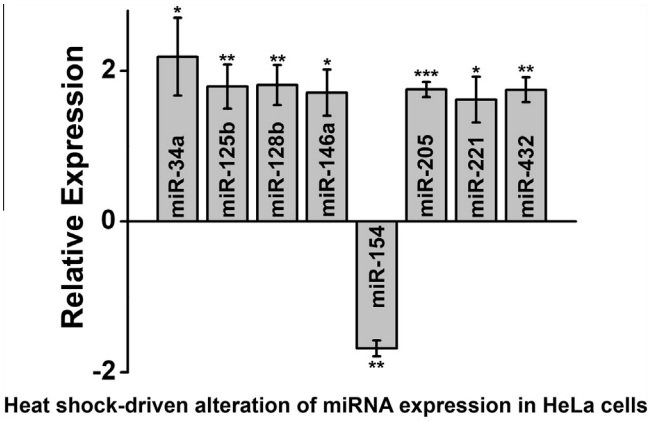

Fig. 1. Altered expression of miRNAs in response to heat shock in HeLa cells. miRNA profiling using qRT-PCR for mature miRNAs using stem-loop miRNA primers in unstressed and stressed HeLa cells. Expression of hsa-miR-17-5p and *β-actin* were taken as endogenous control. Fold change was calculated by considering the normalized expression of miRNAs in unstressed cells (control) as 1. Error bars indicate ± SD. The statistical significance level between various experimental pairs is indicated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

not yet defined, we searched upto 20 kb upstream sequence of pre-miRNAs. It is noteworthy that transcription factors regulating miRNA expression through distal binding sites are already reported [21,22]. One or more putative HSE was identified in upstream sequence of all thermally altered miRNAs, except hsa-miR-146a (Supplementary Table S3). This indicates that HSF1 could regulate miRNA transcription.

3.3. Functional validation of the HSE present upstream of hsa-miR-432

As evident from Supplementary Table S3, minimum distance between a putative HSE and a thermally altered miRNA (pre-miRNA) is 392 bp which separates hsa-miR-432 (pre-miR-432) and a putative HSE present at its upstream region (Fig. 2A). Our next goal was to validate the functionality of the putative HSE. HeLa cells were either kept unstressed or exposed to HS treatment before harvesting and immunoprecipitation was carried out with anti-HSF1 antibody. Immunoprecipitated DNA was eluted and amplified using primer pairs specific for hsa-miR-432 upstream sequence harboring predicted HSF1-binding site. As depicted in Fig. 2B and C, heat shock treatment significantly increased ($p = 0.02$, $n = 3$) occupancy of endogenous HSF1 in its cognate binding site present in hsa-miR-432 upstream sequence.

To check whether the HSE is transcriptionally active, HeLa cells transiently expressing either empty vector or miR-432_ups or Hsp70_ups were either kept unstressed (control) or exposed to standard HS treatment and finally harvested to carry out luciferase assay. Result showed (Fig. 2D) that heat shock treatment significantly increased luciferase activity ($p = 0.007$, $n = 3$) of miR-432_ups construct in HeLa cells. This increase in luciferase activity is believed to be caused by heat shock-driven activation of endogenous HSF1. The Hsp70_ups construct, as expected, showed robust increase in reporter activity in response to heat shock (Fig. 2D).

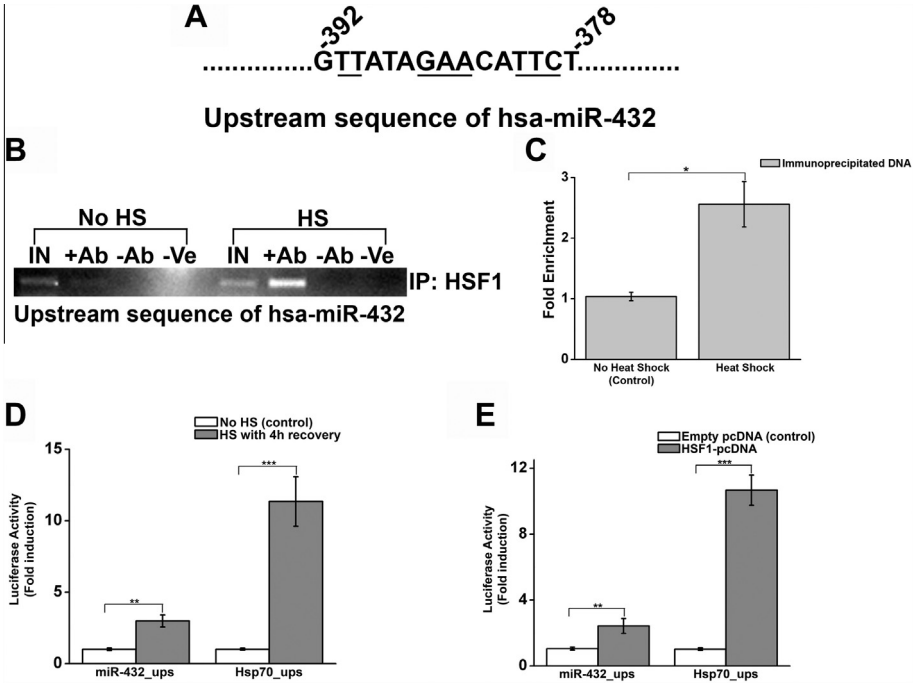

Fig. 2. Functional validation of heat shock element (HSE) present upstream of hsa-miR-432. (A) Presence of HSE in the upstream sequence of hsa-miR-432. Sequence was retrieved from ENSEMBL Biomart. Consensus nucleotides are underlined. (B) ChIP showing *in vivo* interaction of HSF1 with upstream sequence of hsa-miR-432 in HeLa cells subjected to no HS treatment and HeLa cells exposed to HS treatment. Immunoprecipitation was carried out using anti-HSF1 antibody and precipitated DNA was PCR-amplified using primers flanking the HSE present in upstream sequence of hsa-miR-432. Lane IN: PCR amplification was carried out using DNA isolated from HeLa cells subjected to no HS or HS treatment. Lane +Ab: PCR amplification was carried out using chromatin immunoprecipitated by anti-HSF1 antibody. Lane IgG: PCR amplification was carried out using chromatin immunoprecipitated by IgG alone. Lane −Ve: PCR amplification was carried out without any DNA. (C) Quantitative analysis of ChIP assay by normalizing the amount of immunoprecipitated DNA to the input DNA in each sample and fold enrichment was calculated by considering the normalized immunoprecipitated DNA in unstressed cells (control) as 1. (D) Luciferase reporter assay of empty pGL3 vector (control), miR-432_ups and Hsp70_ups in HeLa cells in absence (control) and presence of HS treatment. Luciferase activity of the above cells was normalized by the luciferase activity of the corresponding empty pGL3 vector transfected cells. (E) Luciferase reporter assay of reporter constructs described in D in HeLa cells transiently transfected with empty pcDNA vector (control) and HSF1-pcDNA and exposed to HS treatment. Luciferase activity of the above cells was normalized by the luciferase activity of the corresponding empty pGL3 vector transfected cells. Error bars indicate ± SD. The statistical significance level between various experimental pairs is indicated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

To test the direct effect of HSF1 on reporter activity of miR-432_ups, HeLa cells were transiently transfected with reporter constructs (empty vector or miR-432_ups or Hsp70_ups) along with empty pcDNA vector or HSF1-pcDNA and luciferase assay was carried out in presence of standard HS treatment. As shown in Fig. 2E, cells expressing exogenous HSF1 showed significant increase in luciferase activity ($p = 0.006$, $n = 3$) compared to control cells (cells expressing empty pcDNA vector). Therefore the HSE present in hsa-miR-432 upstream sequence is responsive to both heat shock as well as ectopic HSF1.

3.4. HSF1 regulates hsa-miR-432 expression

We next aimed to determine whether HSF1 could regulate expression of hsa-miR-432. HeLa cells transiently expressing either empty pcDNA vector (control) or HSF1-pcDNA were subjected to standard HS treatment and expression of hsa-miR-432 was measured by qRT-PCR. Exogenous HSF1, upon heat shock, was found to elevate the expression of hsa-miR-432 significantly ($p = 0.03$, $n = 3$) in HeLa cells (Fig. 3A).

To further show the contribution of HSF1 in regulating hsa-miR-432 expression, we wanted to examine whether loss-of-function of HSF1 has any effect on hsa-miR-432 expression. Endogenous HSF1 was knocked-down in HeLa cells by siRNA as described earlier [15]. Quantitative RT-PCR revealed no significant change in endogenous hsa-miR-432 expression upon HSF1 knockdown (Fig. 3B). Next, cells expressing empty pSUPER vector or HSF1-siRNA or scrambled siRNA were subjected to standard HS treatment and hsa-miR-432 expression was measured in each sample by qRT-PCR. As shown in Fig. 3C, HeLa cells expressing either empty pSUPER vector ($p = 0.03$, $n = 3$) or scrambled RNA ($p = 0.03$, $n = 3$) showed increased hsa-miR-432 expression following heat shock, whereas no significant change in hsa-miR-432 expression was observed in HSF1-knocked down cells. Therefore, knocking down endogenous HSF1 reduced the ability of HeLa cells to increase miR-432 expression in response to heat stress.

3.5. Enrichment analysis with targets of thermally altered miRNAs

Finally we carried out an enrichment analysis with targets of all thermally altered miRNAs to reveal the probable functions of these miRNAs. Validated targets of 8 thermally altered miRNAs were collated from miRTarBase [23,24] which revealed that hsa-miR-34a, hsa-miR-125b, hsa-miR-128b, hsa-miR-146a, hsa-miR-154, hsa-miR-205, hsa-miR-221 and hsa-miR-432 are known to target 495, 289, 4, 66, 1, 22, 249 and 4 genes respectively (Supplementary Table S4). Altogether these 8 thermally altered miRNAs target 1059 unique genes. Enrichment analysis of various Gene Ontology (GO) terms with these 1059 genes was performed using GeneCodis software [25–27]. As depicted in Supplementary Table S5, 702 different GO biological processes, 154 different GO molecular function classes and 103 different KEGG pathways were significantly enriched (corrected hypergeometric significance of $\leqslant 0.05$) with genes that are targeted by thermally altered miRNAs.

Significantly over-represented GO biological processes included positive regulation of transcription from RNA polymerase II promoter (GO:0045944), apoptotic process (GO:0006915), cellular response to hypoxia (GO:0071456) etc. Significantly enriched GO molecular functions included protein binding (GO:0005515), DNA binding (GO:0003677), transcription factor binding (GO:0008134), chaperone binding (GO:0051087) etc. KEGG pathways that were significantly enriched included various types of

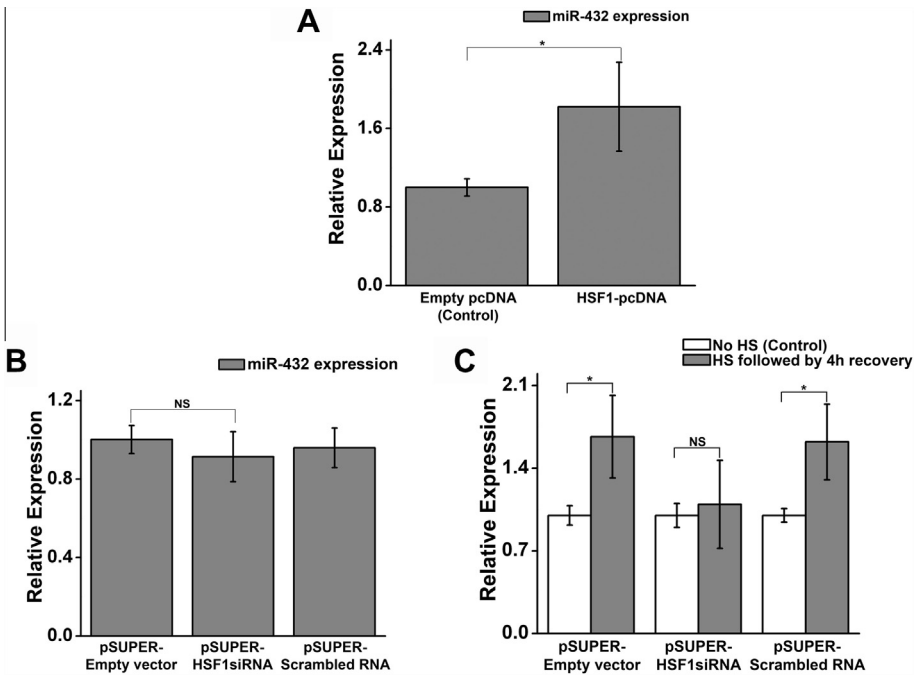

Fig. 3. HSF1 regulates hsa-miR-432 expression in HeLa cells. (A) Bar graph showing relative expression of hsa-miR-432 measured by qRT-PCT in empty vector (pcDNA) transfected cells (control) and HSF1-pcDNA transfected cells both exposed to HS treatment. Expression of hsa-miR-17-5p and *β-actin* were taken as endogenous control. Relative expression of hsa-miR-432 was calculated by considering the normalized expression hsa-miR-432 in control cells as 1. (B) Bar graph showing relative expression of hsa-miR-432 measured by qRT-PCT in pSUPER vector transfected (control), pSUPER-HSF1-siRNA transfected and pSUPER-scrambled RNA transfected cells. Expression of hsa-miR-17-5p and *β-actin* were taken as endogenous control. Relative expression of hsa-miR-432 was calculated by considering the normalized expression hsa-miR-432 in control cells as 1. (C) Bar graph showing three independent experiments for qRT-PCR of hsa-miR-432 expression in cells transiently transfected with pSUPER vector, pSUPER-HSF1-siRNA and pSUPER-scrambled RNA and subjected to HS treatment 72 h after transfection. Expression of hsa-miR-17-5p and *β-actin* were taken as endogenous control. Relative expression of hsa-miR-432 was calculated by considering the normalized expression hsa-miR-432 in control cells as 1. Error bars indicate ± SD. The statistical significance level between various experimental pairs is indicated ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

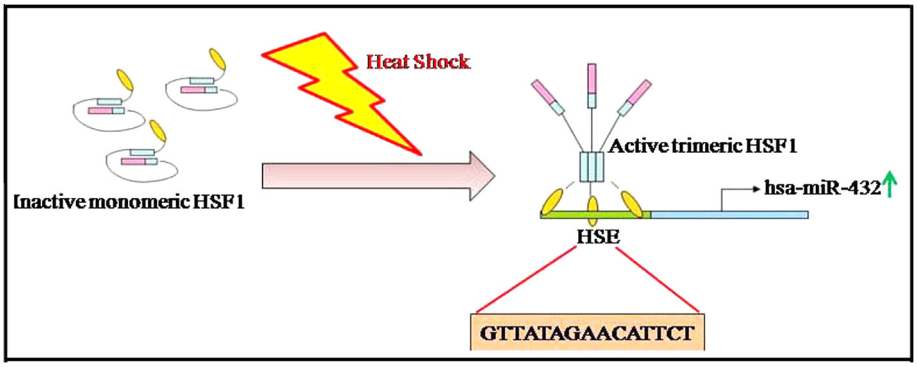

Fig. 4. Proposed model showing regulation of hsa-miR-432 expression by heat shock factor 1. Upon heat shock, inactive monomeric HSF1 is converted to transcriptionally competent active trimeric form which then binds to the HSE present in upstream sequence of hsa-miR-432 thereby induces its expression.

cancer pathways (prostate cancer, pancreatic cancer, chronic myeloid leukemia etc.), pathways associated with protein folding diseases (Huntington's disease, Alzheimer's disease, Parkinson's disease etc.), apoptosis etc. Complete list of enriched GO terms and KEGG pathways have been given in Supplementary Table S5.

4. Discussion

In the present communication we reported heat shock-driven alteration of 8 miRNAs in HeLa cells. We further showed that hsa-miR-432 expression is regulated by HSF1 in heat shock-dependent manner through a functionally active HSE present in upstream sequence of the miRNA. To the best of our knowledge this is the first report showing direct involvement of HSF1 in regulating miRNA expression and hsa-miR-432 is a novel transcriptional target of HSF1.

Altered expression of miRNAs in response to heat stress have previously been reported in human [13], mice [14,28] and rat [29]. We measured expression of 38 miRNAs chosen from earlier study [18], published literatures and available ChIP-seq data, in cells exposed to HS treatment and identified alteration of 8 miRNAs in HeLa cells (Fig. 1). hsa-miR-17-5p expression was unaltered by heat shock and therefore we used hsa-miR-17-5p as endogenous control, as also used previously [18,30]. In addition, *β-actin* expression was used as second endogenous control in heat shock experiments. Among the 7 miRNAs upregulated in our study, hsa-miR-34a and hsa-miR-125b have previously been shown to be upregulated by heat shock in human dermal fibroblasts [13]. The same study also reported downregulation of hsa-miR-154 in response to hyperthermia [13], as also observed by us. Increased expression of miR-34a by thermal stress has also been observed in rat [29].

Recently hsa-miR-432 has been shown to induce neuronal differentiation [31] in human neuroblastoma cells however no information is available regarding the regulation of hsa-miR-432 expression. From the observation that expression of hsa-miR-432 and its host gene has no correlation, Marsico et al. postulated that hsa-miR-432 is expected to be regulated via regulatory sites independent of its host gene promoter [32]. We identified and subsequently validated the functionality of the HSE present in hsa-miR-432 upstream sequence (Fig. 2). The observation that overexpression of HSF1 increased hsa-miR-432 expression in presence of heat shock and knocking down endogenous HSF1 inhibited the ability of cells to induce heat shock-mediated hsa-miR-432 expression (Fig. 3) further indicates that HSF1 is a regulator of hsa-miR-432 expression in HeLa cells. Combining our results, we propose a working model depicting regulation of hsa-miR-432 expression by HSF1 (Fig. 4).

hsa-miR-432 is member of a cluster that includes hsa-miR-136, hsa-miR-127, hsa-miR-431, hsa-miR-433 etc. Of these miRNAs, hsa-miR-127 was reported to be induced in a urinary bladder carcinoma cell line and in fibroblasts, independent of the other members of the same miRNA cluster via a transcription start site located a few hundred base pairs upstream of the pre-miRNA [33]. It is noteworthy that unlike hsa-miR-432, hsa-miR-127 was found unaltered by heat shock in our study (Supplementary Table S2). Our findings thus suggest that depending on cell type and growth condition, members of this miRNA cluster might be regulated independently. We however don't rule out the possibility of presence of other regulatory sites of HSF1 and/or other transcription factors that might regulate hsa-miR-432 expression. Furthermore it would be tempting to examine the functionality of other putative HSEs identified bioinformatically in upstream sequences of thermally altered miRNAs (Supplementary Table S3).

Enrichment analysis of various GO molecular functions, GO biological processes and KEGG pathways with validated targets of thermally altered miRNAs revealed a functional correlation between HSF1 and heat shock-induced or repressed miRNAs. GO molecular function and biological processes involving transcription regulation, signaling, apoptosis, stress response etc. which have long been associated with HSF1, were significantly enriched (Supplementary Table S5). Similarly KEGG pathways those were significantly over-represented amongst the targets of thermally altered miRNAs included pathways known to be regulated by HSF1 like different types of cancer and proteopathies (Supplementary Table S5). This overlapping of function of HSF1 and thermally altered miRNAs at the molecular and cellular level suggests that these miRNAs may play important role as downstream effector molecule of HSF1 and may participate actively in regulating wide range of biological processes important for cell survival or death.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.100.

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