NRF2-driven miR-125B1 and miR-29B1 transcriptional regulation controls a novel anti-apoptotic miRNA regulatory network for AML survival.

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Running Title: NRF2 regulates miR-125B and miR-29B in AML

Abstract

Transcription factor NRF2 is an important regulator of oxidative stress. It is involved in cancer progression, and has abnormal constitutive expression in acute myeloid leukemia (AML). Post-transcriptional regulation by microRNAs (miRNAs) can affect the malignant phenotype of AML cells. In this study we identified and characterised NRF2-regulated miRNAs in AML. A miRNA array identified miRNA expression level changes in response to NRF2-knockdown in AML cells. Further analysis of miRNAs concomitantly regulated by knock-down of the NRF2 inhibitor KEAP1, revealed the major candidate NRF2-mediated miRNAs in AML. We identified miR-125B to be upregulated by NRF2 and miR-29B to be downregulated by NRF2 in AML. Subsequent bioinformatic analysis identified putative NRF2 binding sites upstream of the miR-125B1 coding region and downstream of the mir-29B1 coding region. Chromatin immunoprecipitation (ChIP) analyses showed NRF2 binds to these antioxidant response elements (AREs) located in the 5' untranslated regions of miR-125B and miR-29B. Finally, primary AML samples transfected with anti-miR-125B antagomiR or miR-29B mimic showed increased cell death responsiveness either alone or co-treated with standard AML chemotherapy. In summary, we find that NRF2 regulation of miR-125B and miR-29B acts to promote leukaemic cell survival, and their manipulation enhances AML responsiveness towards cytotoxic chemotherapeutics.

Keywords and Abbreviations

Keywords: Leukaemia, miRNA, NRF2, cell death, chemotherapy

Abbreviations: Acute myeloid leukemia (AML); Nuclear factor (erythroid-derived 2)-like 2 (NRF2); heme oxygenase-1 (HO-1); NAD(P)H dehydrogenase quinone 1 (NQO1); NRF2 lentiviral knockdown (NRF2-KD); negative control knockdown (NEG-KD); chromatin immunoprecipitation assay (ChIP); microRNAs (miRNAs)

Introduction

Acute myeloid leukemia (AML) is a biologically heterogenous disorder that occurs as a consequence of a wide variety of genetic abnormalities in haematopoietic progenitors that are derived from the bone marrow [1]. However, it is likely that AML share various survival pathways downstream of the driver mutations, making the existence of common survival pathways and therapeutic targets likely [2].

Nuclear factor (erythroid-derived 2)-like 2 (NRF2) is a member of the Cap 'n' Collar basic leucine zipper transcription factor family which protects cells from reactive oxygen species (ROS) through the regulation of a number of cytoprotective genes including heme oxygenase-1 (HO-1) and NAD(P)H dehydrogenase quinone 1 (NQO1) [3-5]. In cancer NRF2 activation is pro-tumoral in a spectrum of malignancies through mutations in NRF2 or its cytosolic inhibitor KEAP1 [6-9]. However we have previously shown that in human AML constitutive activation of the NRF2 signalling pathway is not through somatic mutations of NRF2/Keap1 but as a consequence of upstream constitutive activation by NF-κB [10]. [11]. In addition to contributing to the malignant phenotype of AML, we also found that NRF2 contributes to intrinsic leukaemia cell resistance to standard front-line chemotherapy agents [12]. [10].

miRNAs are short non-coding RNA molecules, approximately 20-30 nucleotides in length, that can post-transcriptionally regulate gene expression by binding to the 3' UTR of their target mRNA, thereby repressing gene transcription [13]. miRNA expression has shown to be dysregulated in a range of cancers, and can act as tumour suppressor miRs (by down-regulating oncogenes), or oncomiRs (by targeting tumour suppressors) [14]. In light of their role in cancers, miRNA are being increasingly studied with the expectation that modulation of their expression can be harnessed to improve cancer therapy. A recent study using chromatin immunoprecipitation sequencing (ChIP-Seq) experiments on lymphoid cells identified high confidence ChIP-Seq peaks for NRF2 binding in the vicinity of several miRNAs, suggesting they could be regulated by NRF2 [15].

In the present study we look to identify whether NRF2 regulates miRNA in human AML. Furthermore we evaluate whether NRF2 regulated miRNA have functional

importance not only on the biology of the disease but also in the leukemia cell death to chemotherapy treatment.

Results

Lentiviral NRF2 knockdown alters miRNA expression

NRF2 can regulate drug resistance in AML via the induction of cytoprotective and detoxification genes [10, 12]. To assess whether NRF2 regulates miRNA expression in AML we used a commercially available OncomiR collection Cancer miRNA qRT-PCR Array on THP-1 cells infected with an NRF2 lentiviral knockdown (NRF2-KD) and a negative control knockdown (NEG-KD). Figure 1A shows the change in expression of miRNA in response to NRF2-KD compared to the control infected NEG-KD. We then treated THP-1 cells with the NRF2 activator, sulforaphane for 6 hours for comparison. Supplementary table 1 shows the fold increase of miRNA in response to both NRF-KD and sulforaphane treatment. From these experiments we identified miR-125B, miR-222, miR-221 and miR-223 as potential miRNA up-regulated by NRF2 and miR-29B and miR-154 as potential miRNA down-regulated by NRF2. Figure 1C shows Western blot analysis of NRF2 and KEAP1 protein expression in THP-1 cells in response to NRF2-KD, KEAP1-KD and NEG-KD.

Increased NRF2 activity increases miR-125B and decreases miR-29B expression in AML

We used a qRT-PCR approach to validate the data from the lentiviral array experiments [10]. THP-1 with NRF2-KD were assayed for miR-125B, miR-221, miR-222, miR223, miR-29B and miR-154 expression. NRF2-KD results in THP-1 cells showing a consistent decrease in miR-125B expression and increasing miR-29B expression as well as smaller but not significant fluctuations in miR-221, miR-222, miR-223 and miR-154 expression (Figure 1B). To further assess the role of NRF2 in regulating miRNA we increased the activity of NRF2 in THP-1 cells by knockdown of the NRF2 inhibitor KEAP1 [16]. THP-1 were infected with KEAP1 lentiviral knockdown (KEAP1-KD). QRT-PCR confirmed the downregulation of KEAP1 and as a positive control we showed that KEAP-KD up-regulated the NRF2 target gene HO-1. Moreover, miR-125B expression showed a similar increase to HO-1 and miR-29B showed a significant decrease in response to KEAP1-KD (Figure 1B). The expression of miR-221, miR-222, miR223 and miR-154 were not significantly changed in response in NRF2-KD or KEAP1-KD to warrant its inclusion in further experiments. Taken together these data identified miR-125B and miR-29B as NRF2 regulated miRNA in AML.

NRF2 regulates homologs miR-125B1 and miR-29B1 and miR-29A in human AML

Both miR-125B and miR-29B exist as two homologs (Figure 2A). miR-125B1 (located on chromosome 11) and miR-125B2 (located on chromosome 21), both contain identical seed sequences. Both homologs lie in the region of regulatory miRNA clusters (miR-100-let-7-a-2) and (LINC00478) respectively, raising the possibility that they are regulated or co-regulated by these clusters. miR-29B also exists as two homologs, miR-29B1 (located on chromosome 7) and miR-29B2 (located on chromosome 1) (Figure 2A).

The sequence of the miRNA seed region of both miR-125B1 and 2 is identical. Similarly the seed sequence of miR-29B1 is identical to miR-29B2. Therefore we used primers designed specifically for the immature miRNA sequences (which does differ between the miRNA homologs) to identify which miR-125B and miR-29B homologs were being regulated by NRF2. QRT-PCR on lentiviral NRF2-KD THP-1 cells identified reduced expression of the immature miR-125B1 and increased expression of the immature miR-29B1, but no change in the expression of immature miR-125B2 and miR-29B2 sequence (Figure 2B and 2C). This suggests that miR-125B1 and miR-29B1, but not miR-125B2 or miR-29B2, are regulated by NRF2.

To determine whether NRF2 specifically regulated miR-125B1 and miR-29B1 or had a more general regulatory effect on their clusters we looked for a differential expression on other miRNAs in each of the homolog regions in response to NRF2-KD. Following NRF2 knock down however there was no difference in the expression of miR-100 and let-7a-2 (miR-125B1 cluster; chromosome 11) or miR-99a and let7c (miR-125B2 cluster; chromosome 21) (Figure 2B). This implies that NRF2 does not regulate these clusters to control miR-125B expression but instead binds to a miR-125B specific regulatory region. Conversely knockdown of NRF2 elevated miR-29B1 and miR-29A but had no effect on miR-29B2 and miR-29C (Figure 2C). NRF2 therefore regulates a defined pattern of expression of the miR-125B1 and miR-29B1 cluster in human AML. Moreover, results from Figure 1B and Figure 2B and C show

that changes in total levels of miR-125B and miR-29B are similar to changes in miR-125B1 and miR-29B1. The main reason for this similarity is that constitutive levels of miR-125B2 and miR-29B2 are much lower in AML than there homolog, thus changes observed in total miR-125B and miR-29B reflect changes observed in miR-125B1 and miR-29B1 levels.

NRF2 binds to ARE sites in the promoters of miR-125B1 and miR-29B1

NRF2 acts as a transcription factor by binding to the anti-oxidant response element (ARE) region in the promoter regions of its target genes [17], therefore we hypothesised that for NRF2 to regulate miR-125B1 and mir-29B1 an ARE site must be present in their regulatory regions. To analyse the 5' miR-125B1 and miR-29B1 promoter sequence we used transcription factor analysis programmes (http://www.gene-regulation.com/cgi-bin/pub/programs /pmatch/bin/p-match.cgi and www.genomatix.de/matinspector.html). These analyses identified three potential ARE sites within the miR-125B1 promoter and two in the miR-29B1 promoter (Figure 3A).

To confirm if NRF2 bound to ARE binding sites in the miR-29B1 or the miR-125B1 promoter a chromatin immunoprecipitation assay (ChIP) was undertaken. Figure 3B shows the analysis using antibodies for NRF2 and quantified against control IgG with specific primers for ARE1, ARE2 and ARE3 (promoter region of miR-125B1; chromosome 11) and ARE4 and ARE5 (promoter region of miR-29B1; chromosome 7). Recruitment of NRF2 was markedly enhanced to ARE5 in the miR-29B1 promoter and ARE3 in the miR-125B1 promoter. Next we evaluated the recruitment of NRF2 to ARE1, ARE2, ARE3, ARE4 and ARE5 sites in THP-1 cells transfected with KEAP1 siRNA. Figure 3C shows that there was increased recruitment of NRF2 to the ARE3 and ARE5 site, but not the other potential ARE sites in THP-1 cells transfected with KEAP siRNA over control siRNA. These observations confirmed that NRF2 specifically binds the miR-29B1 ARE5 site and the miR-125B1 ARE3 site.

To establish whether NRF2 functionally controls miR-125B1 expression the miR-125B1 promoter was cloned into a PGL4 luciferase plasmid and the putative ARE3 site was mutated (Figure 3D). AML is a disease characterised by constitutive

activation of NRF2, therefore the luciferase vector PGL4 and PGL4/p125B were transfected into THP-1 cells without prior treatment of an NRF2 activator or repressor. We observed an approximate 8 fold increase in luciferase activity in the PGL4/p125B plasmid in comparison to the control (Figure 3E). Site directed mutagenesis of the ARE3 site in the miR-125B1 promoter corresponded with a significant decrease in miR-125B1 promoter activity (Figure 3E). Moreover when we co-transfected KEAP1 and NRF2 siRNA with the p125B and p125BNRF2Mut plasmids, KEAP1 siRNA induced significant increase in p125B promoter activity and NRF2 siRNA inhibited p125B promoter activity. KEAP1 siRNA and NRF2 siRNA had no effect on p125BNRF2Mut promoter activity (Figure 3F). Similar experiments on the miR-29B1 promoter were technically not possible as the construct was too big and would not clone into a luciferase reporter vector. These results demonstrate that the ARE3 site in the promoter of mir-125B1 is regulated by NRF2.

NRF2 regulates the expression of miR-125B1 and miR-29B1 in primary human AML

As we have established in AML that high NRF2 mRNA expression causes an increase in NRF2 activity [10], and that miR-125B1 and miR-29B1 are regulated by NRF2 in AML cell lines we next examined the expression of NRF2, mir-125B1 and miR-29B1 in primary human AML cells. NRF2 and miR-125B1 is increased in AML compared to normal CD34+ haematopoietic stem cells (HSC), whereas miR-29B1 is decreased in AML compared to normal CD34+ HSC (Figure 4A). As total levels of miR-125B and miR-29B most likely determine their biological function we analysed total levels of miR-125B and miR-29B in AML. Supplementary Figure 2 shows that total miR-125B is increased in AML compared to normal CD34+ haematopoietic stem cells (HSC), whereas miR-29B is decreased in AML compared to normal CD34+ HSC. Furthermore there is a positive correlation between NRF2 RNA expression and mir-125B1 RNA expression, with an inverse correlation between NRF2 RNA expression and miR-29B1 RNA expression in primary AML cells (Figure 4B). To confirm that NRF2 regulates miR-125B1 and miR-29B1 expression in primary human AML cells we used NRF2 siRNA to silence NRF2 RNA expression in 8 AML patient samples. NRF2 mRNA is significantly knocked down in 7 out of the 8 AML samples of which the majority also had significantly lower miR-125B1 expression (Figure 4C). Moreover, NRF2 siRNA in the AML samples increased miR-

29B1 expression (Figure 4C). Together these results show that knockdown of NRF2 regulates the expression of miR-125B1 and miR-29B1 in primary human AML.

miR-125B antagomiR and miR-29B mimic increases AML apoptosis and sensitivity to frontline chemotherapy agents

Previous studies in AML have suggested that the overexpression of miR-125B plays an oncogenic role by repressing apoptosis and the reduction of miR-29B increases proliferation and represses apoptosis [18] [19]. To determine the functional role of inhibiting miR-125B and overexpressing miR-29B both alone and in-combination we transfected miR-125B antagomiR and miR-29B mimic into THP-1 cells and analysed cells for apoptosis by annexin V/PI staining. However, before we did this we showed that the concentrations of transfected miR-125B antagomiR and miR-29B mimic used manipulated the miRNA levels comparable to that observed in the NRF2-KD and KEAP1-KD THP-1 cells (supplementary Figure 1). THP-1 cells transfected with the miR-125B antagomiR or miR-29B mimic showed a small but significant increase in annexin V/PI staining in comparison to the control transfection (Figure 5A). Moreover, when we co-transfected miR-125B antagomir and miR-29B mimic we observed a synergistic increase in annexin V/PI staining in comparison to the control (Figure 5A).

Transfection of miR-125B antagomir and miR29B mimic into THP-1 and Kasumi-1 cells potentiated the cytotoxic effect of the frontline AML chemotherapy agent daunorubicin. We observed a significant decrease in the IC50 value for daunorubicin in miR-125B and miR-29B transfected cells compared to control cells (Supplementary Table 2). Together these results show that miR-125B antagomiR together with miR-29B mimic can increase apoptosis of AML cell lines as well as increase their sensitivity to AML chemotherapy.

Finally we examined the effects of inhibiting miR-125B and increasing miR-29B expression in primary AML cells in comparison to non-malignant primary CD34+ HSC. In 6 primary AML patient samples inhibition of miR-125B or increasing miR-29B showed a significant decrease in cell viability (Figure 5B). Moreover, we also observe a much bigger increase in AML apoptosis when AML cells were transfected with miR-125B antagomiR together with a miR-29B mimic. A slight decrease in

CD34+ HSC viability was observed in response to miR-125b antagomiR in combination with miR-29B. Finally we compared the viability of primary AML patient cells which had normal NRF2 expression (and thus normal miR-125B and miR-29B expression) to an AML patient sample which had high NRF2 expression (and thus high miR-125B and low miR-29B expression) after treatment with daunorubicin (Figure 5C and Table 1 and Supplementary Table 2). AML with normal NRF2 and normal miR-125B and miR-29B expression were much more sensitive to daunorubicin than AML patient samples which had high NRF2 expression and thus high miR-125B and low miR-29B expression. Moreover, when we transfected the primary AML that had high NRF2 and high mir-125B and low miR29B with miR-125B antagomiR together with miR-29B mimic the result was that sensitivity of these AML cells to daunorubicin was increased to a level comparable to AML with normal NRF2 expression. Since AML#27 has low NRF2 expression over normal CD34+ HSC (Table 1), is more sensitive to daunorubicin (Figure 5C), and miR-125B antagomiR together with miR-29B mimic had no effect on resistance to daunorubicin (Figure 5C), we examined the effect of KEAP1-KD on AML#27 to determine if we could increase the activity of NRF2 and increase the chemoresistance of this sample. Figure 5D shows that KEAP1-KD in AML#27. Figure 5E shows that that KEAP1-KD AML#27 is more resistant to daunorubicin compared to NEG-KD. However when we transfected AML#27 KEAP1-KD cells with control miRNA, miR-125B antagomiR, miR-29B mimic and miR-125B antagomiR in combination with miR-29B mimic, only the combination decreased resistance to daunorubicin (Figure 5F). These results demonstrate that inhibiting miR-125B and overexpressing miR-29B in combination increases apoptosis of primary AML and also increases sensitivity of AML cells to current frontline chemotherapy.

miR-29B and miR-125B gene targets in human AML

Studies have shown that a number of different targets exist for both miR-29B and miR-125B in AML (Supplementary table 3). Moreover, some genes are targeted by both miR125B and miR29B including CDK6, BCL2, MCL1 and BAK1 [18, 20, 21]. Here we examine the expression of genes listed in Supplementary table 3 for their response to transfection of control miRNA, miR-125B antagomiR, miR-29B mimic and miR-125B antagomiR in combination with miR-29B mimic. From the miR-29B targets Figure 6A shows that MYBL2, AKT2, CDK4 and SP1 are inhibited by miR-

29B mimic in THP-1 cells, however we also observe that AKT2 and CDK4 are further inhibited by miR-125B antagomiR in combination with miR-29B mimic. From the miR-125B targets Figure 6A shows that MAPK14 and STAT3 show an increase in mRNA in response to miR-125B antagomiR. We observe a significant increase in BAK1 mRNA expression in response to miR-125B antagomiR, which is not effected by the addition of miR-29B mimic. Finally we observe a decrease in BCL2 expression in response to miR-29B mimic which is slightly reversed by the addition of miR-125B antagomiR. To determine if the effects observed in mRNA was translated into protein in THP-1 cells transfected with miR-125B antagomiR, miR-29B mimic and miR-125B antagomiR in combination with miR-29B mimic we examined AKT2, STAT3 and BAK1. Figure 6B shows a reduction in AKT2 protein expression in response to miR-29B mimic and miR-125B antagomiR in combination with miR-29B mimic. STAT3 showed a slight increase in response to miR-29B mimic and miR-125B antagomiR in combination with miR-29B mimic. BAK1 showed an increase in response to miR-125B antagomir and miR-125B antagomiR in combination with miR-29B mimic. Together these results show that mir-29B and miR-125B regulate a number of genes involved in proliferation and apoptosis in AML cells.

Discussion

Our understanding of the role of non-coding RNA molecules in diseases such as cancer is presently in its infancy. To date miRNA have been shown to have a significant role in cancer biology through the regulation of both tumour suppressors and oncogenes. Less is known however about the upstream control of miRNA expression in cancer and the biological processes which control miRNA expression. In AML differential expression of a number of miRNA has been reported to be associated with the malignant phenotype but as yet the upstream mechanisms by which this is controlled have yet to be described. As we have previously identified NRF2 as an important regulator of cell survival in AML the aim of the present study was to investigate the ability of NRF2 to effect miRNA expression and furthermore define the function of these miRNA in human AML both in a resting state and in response to front line chemotherapeutic agents.

Through gene expression data and multiple transcription factor binding assays we found that NRF2 up-regulates mir-125B1 and down regulates miR-29B1 though binding to specific ARE sites in the miR-125B1 and miR-29B1 regulatory regions. Furthermore we observed that by decreasing the expression of miR-125B and increasing the expression of miR-29B the cells become more sensitive to apoptotic stimuli. These findings are in keeping with previous observations that miR-29B and miR-125B regulate the expression of multiple survival pathways in AML. Specifically miR-125B acts as an oncomiR in AML by down-regulating various tumour suppressor genes including BAK1, BMF and STAT3 [20-22]. miR-29B act as a tumour suppressor by inhibiting the expression of pro-survival genes including AKT2, BCL2, CDK4 and SP1[23-25]. The global repression of miR-125B targets and elevation of miR-29B targets genes would aid the uncontrolled proliferative phenotype of AML. Therefore, in Figure 6A and 6b we analyse the targets of miR-29B and miR-125B in AML cells transfected with corresponding mimic and antagomir, both alone and in combination. We observe changes in a number of genes including AKT2, CDK4, MAPK14, STAT3, BCL2 and BAK1. Interestingly, AKT2 and CDK4 are targets of miR-29B only, however when we co-transfect miR-125B antagomir and miR-29B mimic we do observe a further reduction over miR-29B mimic only. There are no miR-125B sites within these two genes suggesting that the combined observations are due to off target effects. Together the cumulative repression of miR-29B and the over expression of miR-125B significantly contributes to leukemia cell proliferation within the bone marrow niche.

In the clinic we observe that once AML is established the principle cause of refractory or relapsed disease is resistance to our currently used drugs [26]. We have previously shown that high NRF2 activity in AML can protect AML blasts from chemotherapy induced cytotoxicity via the regulation of anti-oxidant genes. Here we extend the chemo-protective role of NRF2 in human AML via regulation of miR-125B and miR-29B, and furthermore explain the previous observations that both high miR-125B and low miR-29B are associated with chemotherapy resistance [21, 27-29]. As we found that miR-125B antagomiR and miR-29B mimic in combination enhance the sensitivity of AML cells to chemotherapy, targeting these miRNA, or NRF2 (the transcription factor which controls there expression), is a biologically plausible novel strategy for AML therapy.

Transcription factors are generally structured in context of networks. In AML miR-29B down regulation has been found to be associated with high C-MYC levels as well as high SP1 and NF-κB [23, 24]. Furthermore NF-κB, JUN and C-MYC have been linked to increased NRF2 activity in human cancer (including AML) [10, 30] and NRF2 has been shown to transcriptionally regulate miR-125B in kidney epithelia cells in response to cisplatin-induced toxicity [31]. Taken together it is likely that NRF2 plays a central role in the complex network regulating miR-29B and miR-125B in human AML.

We also highlight that other miRNA may be regulated by NRF2. The array data shown as supplementary Table 1 shows that some miRNAs are down-regulated in response to NRF2-KD and increased in response to sulforaphane. This list includes miR-222, miR221, miR-223 miR-23a, miR-195 and miR-196a. Of particular interest from this group is miR-222, miR-221 and miR223 which have been shown to be highly expressed in AML compared to other leukaemias [32]. Moreover, we also noted that high NRF2 activity caused a decrease in the expression of miRNA which includes miR-154, miR146a and miR-181B. All these miRNA have been shown to be of interest in AML with miR-146a being shown to have low expression in AML which correlates with high expression of its target gene CXCR4 [33], which in a separate study has been shown to be regulated by NRF2 in human HSC [34]. Consequently in human AML, NRF2 not only regulates key genes involved in the reaction to oxidative stress but also regulates miRNA which appear to have a broader impact on gene expression and regulation.

In summary here we report the first description of upstream miRNA regulation by NRF2 in a human cancer and its downstream cyto-protective consequences. In doing so we not only provide novel mechanistic insight into the pro-tumoral consequences of the constitutive NRF2 expression in AML, we propose that targeting the NRF2 regulation of these miRNA (or the miRNA directly) would be a biologically plausible therapeutic strategy to increase the effectiveness of chemotherapy in human AML.

Experimental Procedures

Materials

The AML-derived cell lines were obtained from the European Collection of Cell Cultures where they are authenticated by DNA-fingerprinting. In the laboratory they are used at low passage number for a maximum of 6 months post-resuscitation, testing regularly for Mycoplasma infection. All primers were purchased from Invitrogen. AKT2, STAT3 and BAK1 antibodies were purchased from Cell Signalling technology (Cambridge, MA). NRF2 and KEAP1 antibodies were purchased from Santa Cruz Biotechnology. All other reagents were obtained from Sigma-Aldrich (St Louis, MO), unless indicated. To assess whether NRF2 regulates miRNA expression in AML we used commercially available OncomiR collection Cancer miRNA qRT-PCR Array.

Primary AML cell culture

AML cells were obtained from patients bone marrow or blood following informed consent and under approval from the UK National Research Ethics Service (LRECref07/H0310/146). For primary cell isolation, heparinized blood was collected from volunteers and human peripheral blood mononuclear cells (PBMCs) isolated by Histopaque (Sigma-Aldrich, UK) density gradient centrifugation. AML samples that were less than 80% blasts were purified using the CD34 positive selection kit (Miltenyi Biotec) (denoted by * in Table 1). Cell type was confirmed by microscopy and flow cytometry. We obtained hematopoietic CD34+ cells from two sources, Stem Cell Technologies and volunteers. Positive selection of CD34+ cells were isolated from PBMCs using a CD34 positive selection kit (Miltenyi Biotec, Auburn, CA). For all CD34+ experiments at least three different donors were used to obtain the results presented in this paper. Cell type was confirmed by microscopy and flow cytometry.

RNA extraction and real time PCR (RT-PCR)

Total mRNA extraction was carried out using mirVana miRNA isolation kit (Ambion) and reverse transcribed using the miRscript II RT kit (Qiagen). All mature miRNA were normalised to RNU6B and immature miRNA and mRNA were normailsed to GAPDH for qRT-PCR as described previously [35]. qRT-PCR was carried out using SYBR green technology (Qiagen). The samples were preamplified at 95C for 2

minutes, after which was amplified for 45 cycles at 95C for 15 seconds, 60C for 10 seconds and 72C for 10 seconds. The qRT-PCR was performed on the LightCycler480 (Roche).

Virus construction and infection

Lentiviruses containing microRNA sequence miRNA-Nrf2 (5′-TTAATGAGTTCAC TGTCAACT-3'), miRNA-KEAP1 (5'-GTTTTGGCCACTGACTGAC-3') and miR-NEG were constructed and produced as described previously [36]. For transduction THP-1 cells and AML blasts cells were plated onto 12-well plates (5×10^4 cells/well) and infected with lentiviruses (multiplicity of infection of 15) with 8 μg/mL Polybrene™. Transduced cells were analyzed by flow cytometry (Accuri), real-time PCR (Roche) and Western Blotting.

Transfections

AML transfections were carried out with $2x10^6$ cells using the Amaxa Nucleofactor Kit II. Control miR, miR-29B mimic and miR-125B antagomiR, control siRNA, KEAP1 and NRF2 siRNA were purchased from Invitrogen were transfected in at a concentration of 45 nm. For Control miR the miR-mimic negative control and the anti-miR miRNA Inhibitor negative control for single experiments and combined miR-mimic negative control and the anti-miR miRNA inhibitor negative control in combined experiments were used. For all gene expression experiments the cells were incubated for 24 hours post transfection before RNA extraction. For the cell viability assays AML cell were incubated for 48 hours post transfection. For reporter assays 0.5μg of PGL4 reporter and pRL-TK control constructs were co-transfected into THP-1. Transfected cells were incubated for 24 hours before the indicated treatments. For reporter assay cells were treated with Dual-Luciferase reporter assay system (Promega).

Western Blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analyses were performed as described previously. Briefly, whole cell lysates as well as nuclear and cytosolic were extracted and sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation performed [37]. Protein was transferred to nitrocellulose

and Western blot analysis performed with the indicated antisera according to their manufacturer's guidelines.

Cloning of miR-125B promoter construct

To generate the p125B promoter construct containing ARE3 a DNA fragment containing 2.6 kb of the human 125b promoter region was amplified from genomic DNA with PCR and specific primers 5-TGAGAGGAGCGCAACAATG-3′ reverse primer and 5′-AGAAAGGCCACCAAGATTCAC-3′ forward primer. The fragment was cloned into the PGL4.11 basic plasmid (Promega). To generate mutated NRF2 construct (p125B NRF2 MUT) the sense PCR primer used was 5′-GCTGTGGCTGtTTTGTtATTCTCTTTGACTAG-3′. This mutation was introduced with the QuikChange XL Site-Directed Mutagenesis Kit (Agilent).

Cell viability and apoptosis assays

THP-1, Kasumi-1, primary CD34+ HSC and primary AML cells were transfected with control miR (miR-mimic negative control and the anti-miR miRNA Inhibitor negative control for single experiments and combined miR-mimic negative control and the anti-miR miRNA Inhibitor negative control in combined experiments), miR-29B mimic and miR-125B antagomiR and combined miR-29B mimic and miR-125B antagomiR for 24 h. THP-1 cells were analysed for apoptosis using PI/Annexin V staining. THP-1, Kasumi-1, primary CD34+ HSC and primary AML transfected cells were treated with different concentrations of chemotherapy agents and incubated for 48 h then viable cells measured with Cell-Titre GLO (Promega).

Chromatin immunoprecipitation assay

THP-1 cells were transfected with control siRNA or KEAP1 siRNA 24 h before the cells were fixed with 1% formaldehyde in medium for 10 min at room temperature. The sonication conditions were optimized to determine generation of DNA fragments between 300 and 600 base pairs in length. Chromatin was immunoprecipitated with IgG, anti-NRF2 (Cell Signalling). The association of NRF2 was measured by RT-

PCR on immunoprecipitated chromatin using primers spanning the ARE sites described in Figure 3A and supplementary table 4.

Statistical analyses

Student's T test was performed to assess statistical significance from controls. Results with $P < 0.05$ were considered statistically significant (*). Results represent the mean ± SD of 3 independent experiments.

Conflict of interest.

The authors declare no conflict of interest.

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Legends

Figure 1. miRNA profiling of AML cells in response lentiviral NRF2 knockdown. (A) THP-1 cells were transduced with NEG (NEG-KD) and NRF2 (NRF2-KD) targeted miRNA lentiviral constructs. QRTPCR analysis of 92 cancer-associated miRNAs in NRF2-KD THP-1 cells. Values represent change in qRT-PCR cycle threshold normalized to RNU6B (ΔCT). Dashed line indicates no change in expression. A red circle indicates miR-125B, miR221, miR-223, miR222, miR29B and miR154. (B) QRT-PCR of miR-125B, miR221, miR-223, miR222, miR29B, miR154, NRF2, KEAP1 and HO1 in THP-1 cells transduced with NEG-KD, NRF2-KD or KEAP1-KD. Values represent fold change in RNA expression over NEG-KD control. (C) THP-1 were transduced with NEG-KD, NRF2-KD or KEAP1-KD before cells were analysed for NRF2 and KEAP1 using Western blotting. Blots were reprobed for βactin to show sample loading. The numbers under the blots indicate densitometry analysis of the blots using Image J software, and the results are expressed as fold change relative to the NEG-KD control.

Figure 2. NRF2 regulates homologs miR-125B1 and miR-29B1 and miR-29A in human AML. (A) Schematic representation of the miRNA chromosomal positioning of miR-125B (1 and 2) and miR-29B (1 and 2). (B) Total RNA was extracted from THP-1 transduced with NEG-KD and NRF2-KD and examined for miRNA expression including immature miR-125B1, miR-125B2 RNA and HO-1 mRNA expression. (C) Total RNA was extracted from THP-1 transduced with NEG-KD and NRF2-KD and examined for miR-29A and miR-29C expression including immature miR-29B1, miR-29B2 RNA and HO-1 mRNA expression.

Figure 3. NRF2 binds to ARE sites in the promoters of miR-125B1 and miR-29B1. (A) Schematic presentation of ARE binding sequences in the 5' region of miR-29B1 on chromosome 7 and miR-125B1 on chromosome 11. (B) Chromatin immunoprecipitation (ChIP) analysis of the miR-29B1 and miR-125B1 promoter using antibodies against NRF2 and normal rabbit IgG was used as a control. QRTPCR was performed in triplicate on immunoprecipitated DNA and input DNA. Data presented as percent of input. * indicates $P < 0.05$ between the different

treatment groups (C) THP-1 cells were transfected control siRNA and KEAP1 siRNA for 24 h and ChIP performed. Real-time PCR was performed in triplicate on immunoprecipitated DNA and input DNA. Data presented as percent of input. * indicates $P < 0.05$ between the different treatment groups. (D) Schematic representation of mutated miR-125B promoter sequence. (E) THP-1 cells were transiently transfected with 0.5 µg of each promoter construct including control plasmid and pRL-TK for normalization of transfection efficiency. Cell extracts were harvested, and luciferase assays were performed. Values are the means ± S.D., $n = 4$. * indicates $P < 0.01$ of deleted ARE against PGL4 control. (F) Control, KEAP1 and NRF2 siRNA were transfected at the same time as p125b and p125bNRF2 MUT and incubated for 48 h Cell extracts were harvested, and luciferase assays were performed. Values are the means ± S.D., $n = 4$. * indicates $P < 0.01$ of KEAP1 siRNA and NRF2 siRNA against NEG siRNA control.

Figure 4. NRF2 regulates miR-29B1 and miR-125B1 in primary AML. (A) Total RNA was extracted from patient AML blasts (n=18) and CD34+ HSC (n=8). NRF2, miR-29B1 and miR-125B1 RNA expression levels were measured using QRTPCR. (B) Pearson's correlation analysis between miR-125B1 or miR-29B1 and NRF2 mRNA expression in human primary AML cells. (C) Control and NRF2 siRNA were transfected into primary blasts (n=7) for 48 h and RNA extracted. RNA was analysed for NRF2, miR-125B1 and miR-29B RNA expression. Values are the means ± S.D., $n = 3$.

Figure 5. miR-125B antagomiR and miR-29B mimic induces apoptosis of AML cells and increases there sensitivity to AML chemotherapy. (A) THP-1 were transfected with control miRNA, miR-125B antagomiR (α125B), miR-29B mimic (29B mimic) and miR-125B antagomiR in combination with miR-29B mimic for 48 h before cells were analysed for apoptosis by PI/Annexin V staining. (B) AML Blasts (n=6) and CD34+ (n=4) were transfected with control miRNA, miR-125B antagomiR and miR-29B mimic and miR-125B antagomiR in combination with miR-29B mimic for 24 h and then assessed for cell viability by Cell Titer-GLO. (C) AML blasts and CD34+ cells were transfected with control miRNA (miR-mimic negative control and the anti-miR miRNA Inhibitor negative control for single experiments and combined miR-mimic negative control and the anti-miR miRNA inhibitor negative control in

combined experiments), miR-125B antagomiR, miR-29B mimic and miR-125B antagomiR in combination with miR-29B mimic for 24 h before the addition of increasing doses of daunorubicin for 48 h. Cells assessed for viability by Cell Titer-GLO. (D) AML#27 was transduced with NEG-KD and KEAP1-KD for 48 hours before cells before the addition of increasing doses of daunorubicin for 48 h before cells were analysed for KEAP1 using Western blotting. Blots were reprobed for βactin to show sample loading. (E) AML#27 was transduced with NEG-KD and KEAP1-KD for 48 hours before the addition of increasing doses of daunorubicin for 48 h Cells assessed for viability by Cell Titer-GLO. (F) AML#27 was transduced with KEAP1-KD for 48 hours before cells were transfected with control miRNA, miR-125B antagomiR, miR-29B mimic and miR-125B antagomiR in combination with miR-29B mimic for 24 h followed by the addition of increasing doses of daunorubicin for 48 h. Cells assessed for viability by Cell Titer-GLO.

Figure 6. miR-125B antagomiR and miR-29B mimic gene targets in AML cells. (A) THP-1 were transfected with control miRNA, miR-125B antagomiR (α125B), miR-29B mimic (29B mimic) and miR-125B antagomiR in combination with miR-29B mimic for 48 h before cells were analysed for target gene expression using QRT-PCR. (B) THP-1 were transfected with control miRNA, miR-125B antagomiR (α125B), miR-29B mimic (29B mimic) and miR-125B antagomiR in combination with miR-29B mimic for 48 h before cells were analysed for AKT2, STAT3 and BAK1 using Western blotting. Blots were reprobed for βactin to show sample loading. The numbers under the blots indicate densitometry analysis of the blots using Image J software, and the results are expressed as fold change relative to the negative control.

Table 1. AML patient sample information, NRF2 RNA expression and relative miR-125B1 and miR-29B1 RNA expression levels. This table defines the nature of the AML disease including WHO diagnosis and cytogenetics. NRF2, miR125B1 and miR-29B1 RNA expression levels over CD34+ control cells

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Number	Age	Gender	WHO diagnosis	Cytogenetics	% Blasts	Fold NRF2 mRNA over CD34+	Fold RNA over CD34+	
							miR-125B1	miR-29B1
AML#1	49	M	AML with maturation	normal	80	3.35	6.57	0.04
AML#2	39	M	AML with maturation	normal	65*	6.89	12.33	0.11
AML#3	64	M	AML with RUNX1-RUNX1T1	t(8;21)	85	1.26	1.09	1.01
AML#4	92	F	AML with myelodysplasia related changes	not available	70*	1.23	1.70	0.20
AML#5	82	F	AML with MDSrelated changes	deletion 13	85	3.39	3.20	0.07
AML#6	46	F	AML with maturation	+4,+8, t(9;22)	70*	4.99	12.51	0.10
AML#7	66	F	AML with maturation	t(2;12)	65*	1.23	5.22	0.27
AML#8	78	M	AML with MDS related changes	not available	85	4.19	9.85	0.01
AML#9	57	M	AML without maturation	not available	95	4.35	4.59	0.09
AML#10	27	M	AML with RUNX1-RUNX1T1	t(8;21)	60*	4.99	10.93	0.08
AML#11	25	M	AML with maturation	normal	50*	4.81	6.63	0.37
AML#12	61	M	Relapsed AML without maturation	not avaiable	95	1.96	1.07	0.71
AML#13	28	F	Acute Monoblastic and Monocytic Leukaemia	normal	90	4.68	6.52	0.33
AML#14	31	F	AML without maturation	trisomy 8	75*	2.73	6.37	0.03
AML#15	84	M	Acute meyloid leukaemia, NOS	not available	70*	3.99	2.43	0.24
AML#16	53	M	AML with t(6;9)(p23;q34);*DEK-NUP214*	t(6;9)	65*	3.25	9.13	0.24
AML#17	51	F	AML with maturation	normal	40*	4.54	3.45	0.02
AML#18	47	M	Acute myeloid leukaemia without maturation	not avaiable	90	4.20	2.07	1.33
AML#19	77	F	AML with maturation	normal	70*	2.78	7.70	0.21
AML#20	62	M	AML with maturation	complex	55*	2.96	2.67	0.59
AML#21	70	M	AML with minimal differentiation	normal	95	2.01	1.98	0.64
AML#22	65	F	AML with maturation	Normal	40*	1.44	2.75	0.24
AML#23	77	M	Therapy related AML	complex	70*	3.24	3.10	0.24
AML#24	40	F	AML with minimal differentiation	normal	90	1.77	3.23	1.09
AML#25	70	M	AML without maturation	complex	95	3.18	6.54	0.42
AML#26	91	F	AML NOS	not available	75*	1.08	1.63	0.97
AML#27	59	F	AML with t(8;21)(q22;q22); RUNX1-RUNX1T1	t(8;21)	85	1.17	1.44	0.53

Table 1. AML patient sample information, NRF2 RNA expression and relative miR-125B1 and miR-29B1 RNA expression levels. This table defines the nature of the AML disease including WHO diagnosis and cytogenetics. NRF2, miR125B1 and miR-29B1 RNA expression levels over CD34+ control cells

Figure 1 - Shah et al

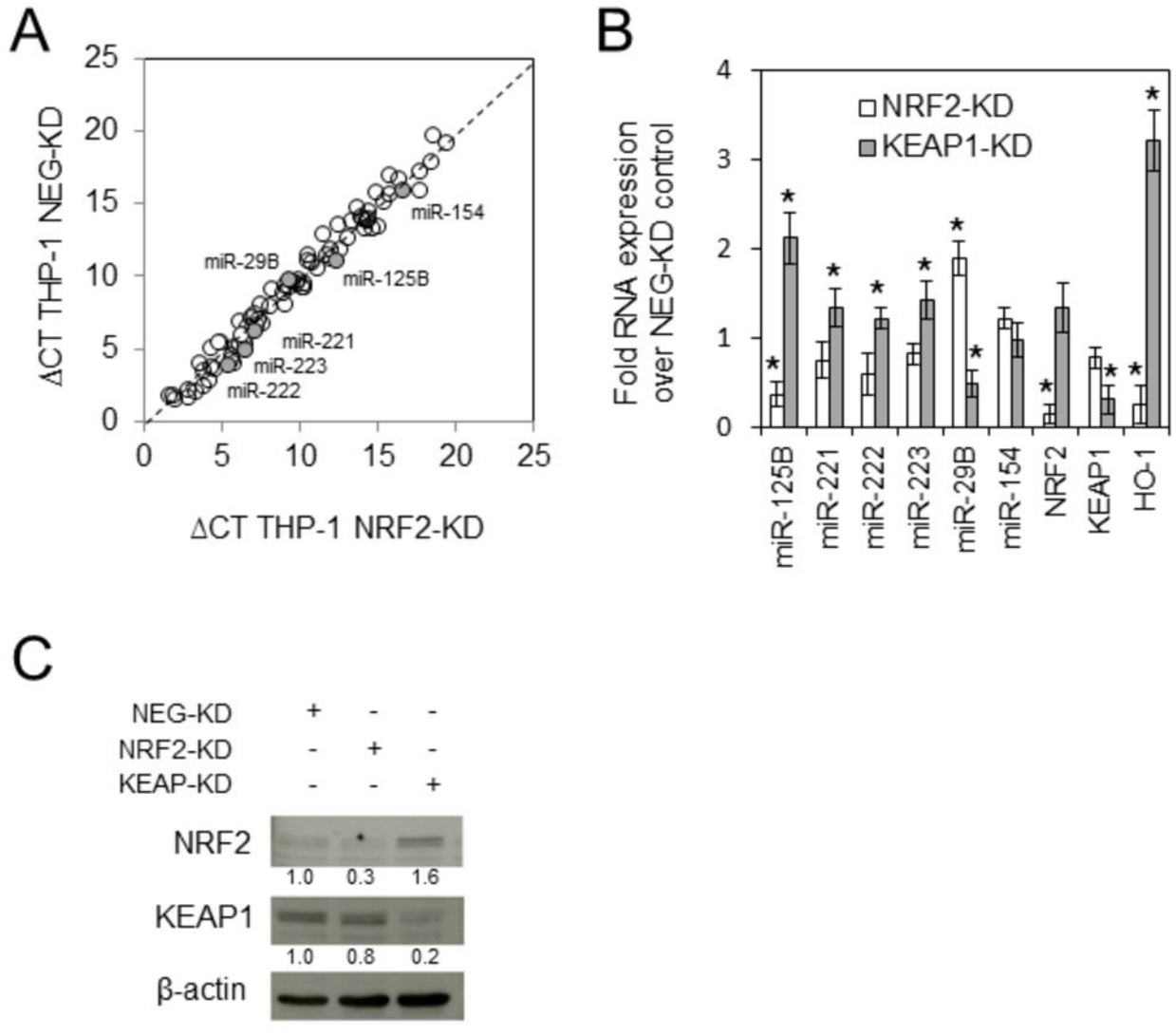
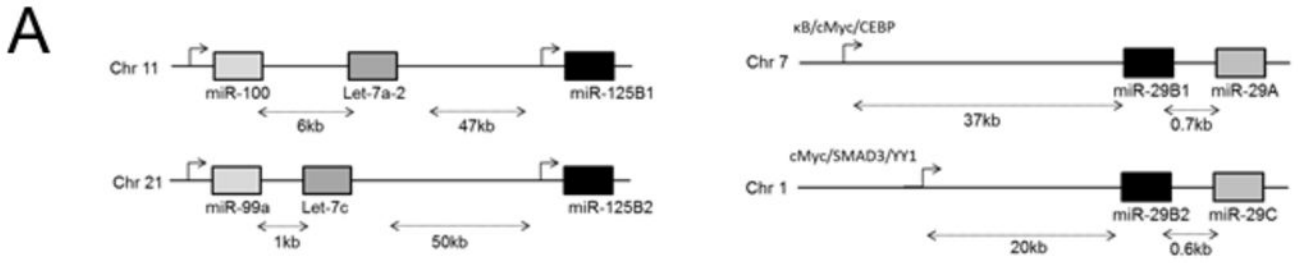
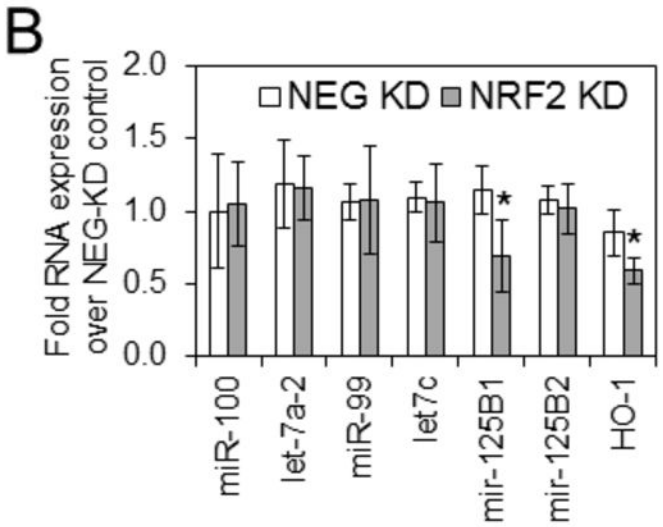


Figure 2 - Shah et al





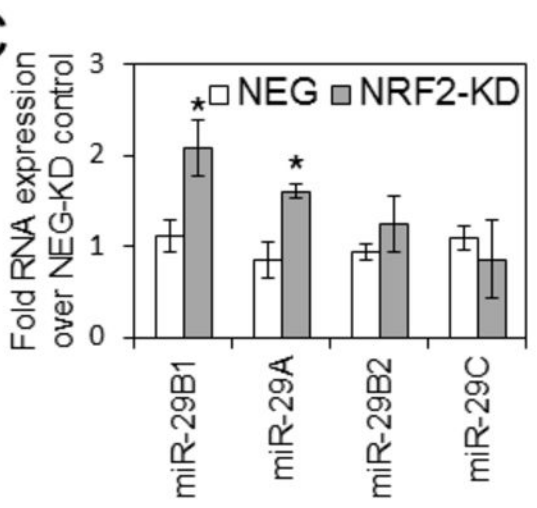


Figure 3 - Shah et al

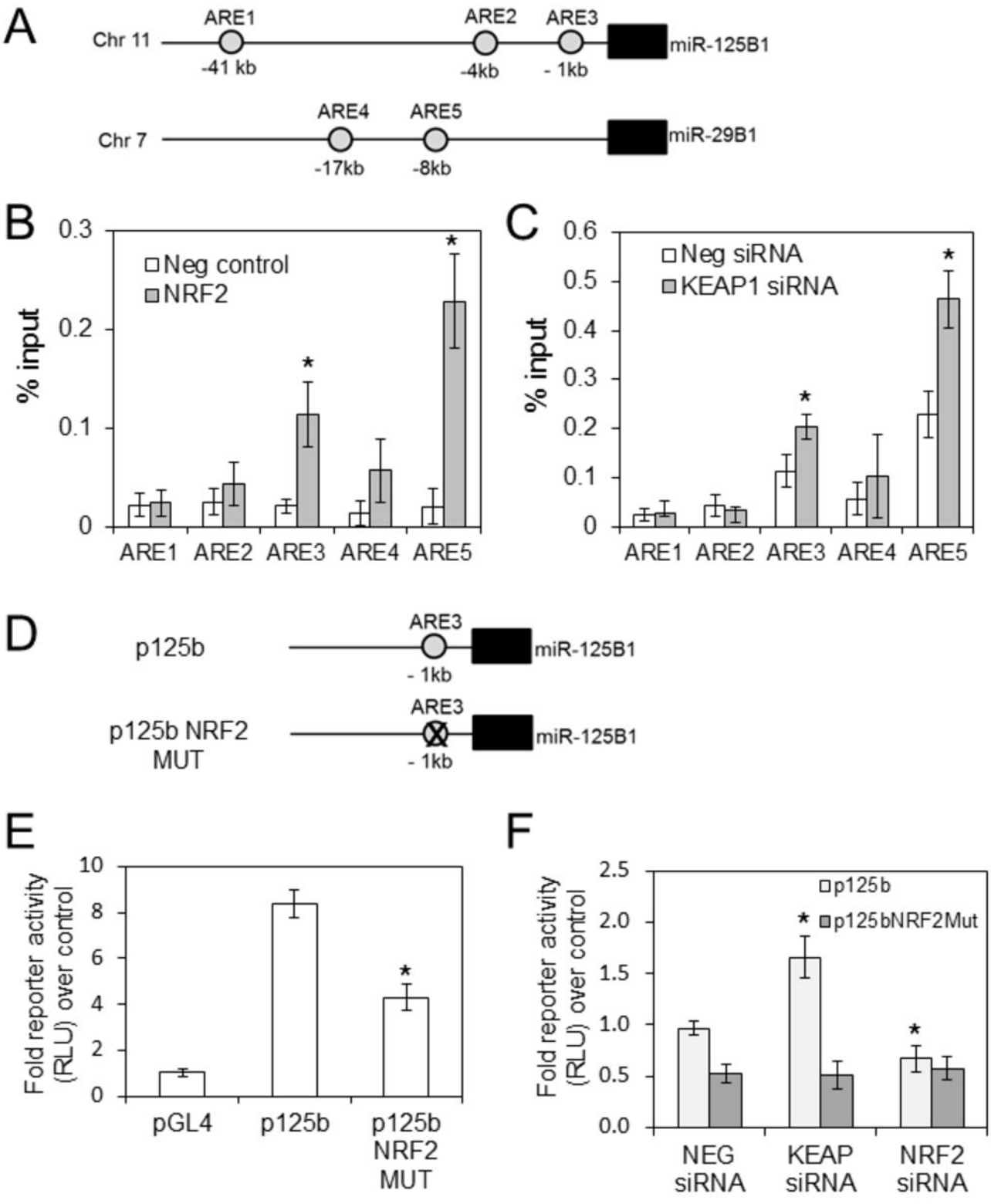


Figure 4 - Shah et al

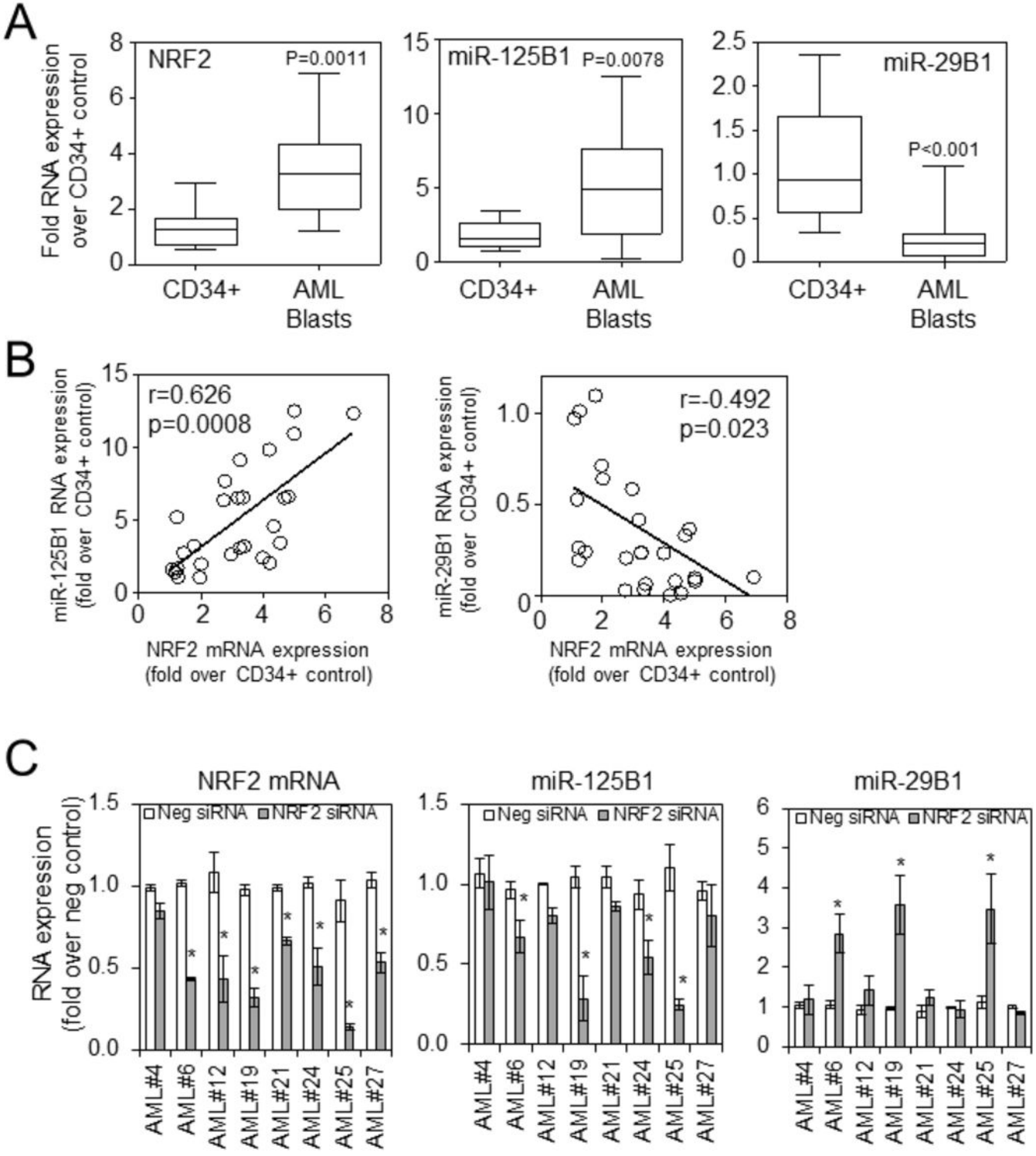


Figure 5 - Shah et al

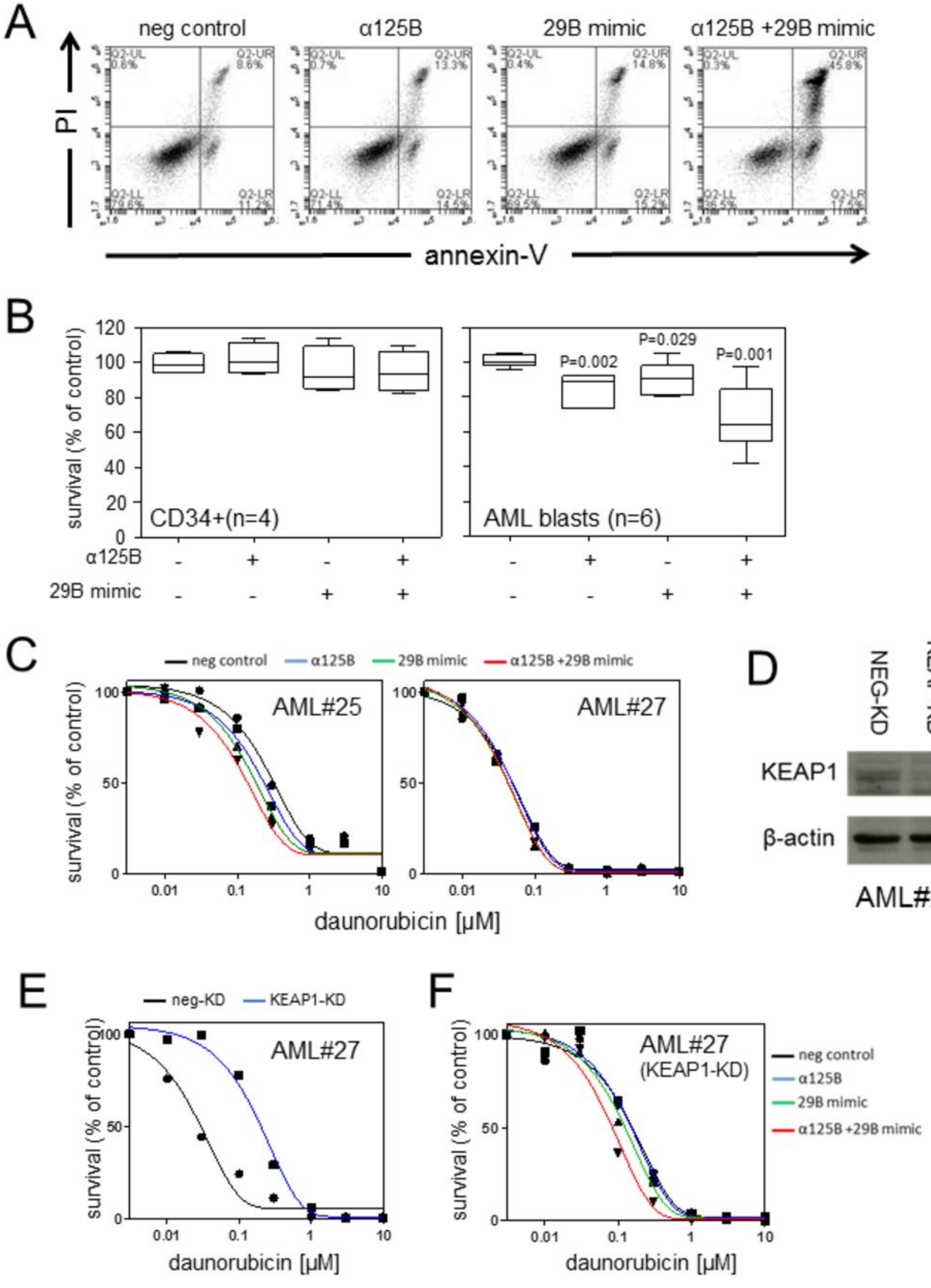


Figure 6 - Shah et al