**Eef1a2 Promotes Cell Growth, Inhibits Apoptosis and Activates JAK/STAT and AKT Signaling in Mouse Plasmacytomas**

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**Abstract**

**Background:** The canonical function of EEF1A2, normally expressed only in muscle, brain, and heart, is in translational elongation, but recent studies suggest a non-canonical function as a proto-oncogene that is overexpressed in a variety of solid tumors including breast and ovary. Transcriptional profiling of a spectrum of primary mouse B cell lineage neoplasms showed that transcripts encoding EEF1A2 were uniquely overexpressed in plasmacytomas (PCT), tumors of mature plasma cells. Cases of human multiple myeloma expressed significantly higher levels of EEF1A2 transcripts than normal bone marrow plasma cells. High-level expression was also a feature of a subset of cell lines developed from mouse PCT and from the human MM.

**Methodology/Principal Findings:** Heightened expression of EEF1A2 was not associated with increased copy number or coding sequence mutations. shRNA-mediated knockdown of Eef1a2 transcripts and protein was associated with growth inhibition due to delayed G1-S progression, and effects on apoptosis that were seen only under serum-starved conditions. Transcriptional profiles and western blot analyses of knockdown cells revealed impaired JAK/STAT and PI3K/AKT signaling suggesting their contributions to EEF1A2-mediated effects on PCT induction or progression.

**Conclusions/Significance:** EEF1A2 may play contribute to the induction or progression of some PCT and a small percentage of MM. Eef1a2 could also prove to be a useful new marker for a subset of MM and, ultimately, a possible target for therapy.

**Introduction**

Cancer is a genetic disease in which tumor cells acquire the ability to proliferate uncontrollably, resist apoptosis, evade immune surveillance, and, for solid tumors, promote angiogenesis. Much of our understanding of tumor initiation and progression has resulted from the identification of genes controlling cell proliferation and apoptosis that, when aberrantly expressed, result in abnormal cell growth and malignant transformation. Considerable attention has been focused on a number of oncogenic signaling pathways that converge on a set of nuclear transcription factors. These factors, in turn, govern the activation of gene expression programs that ultimately result in malignancy. Recently, however, a number of studies have indicated that dysfunctional protein translation may also contribute to tumor development. This is perhaps best exemplified by the roles identified for the protein elongation factor, EEF1A2, in a number of human cancers [1,2,3,4,5,6,7].

EEF1A1 and EEF1A2 are variants of the protein elongation factor EEF1A with EEF1A1 being expressed ubiquitously while EEF1A2 is normally expressed only in heart, muscle and brain [8,9,10]. The canonical role for these proteins involves regulation of ribosomal polypeptide elongation by binding of amino-acylated tRNA for transport to the ribosomes [11]. EEF1A2 has also been found to have a number of non-canonical functions including phosphatidylinositol signaling [12], apoptosis [13,14], cytoskeletal modifications [15,16,17], targeting proteins for degradation, and participation in the heat shock response [18,19]. It has also been shown that EEF1A2 can transform cells and give rise to tumors in nude mice [20]. Notably, EEF1A2 has anti-apoptotic functions in certain systems, whereas EEF1A1 is a pro-apoptotic protein [14,21].

Our interest in EEF1A2 was kindled by results from gene expression profiling of primary mouse B cell lineage tumors that revealed uniquely high expression in plasmacytomas (PCT), neoplasms of mature plasma cells [22,23,24,25]. Our curiosity


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was heightened by the findings that EEF1A2 was also expressed at high levels in some cases of multiple myeloma (MM), a plasma cell neoplasm of humans, but not by normal plasma cells or B cells in either species. Using in vitro model systems, we found that EEF1A2 is involved in regulating cell cycle progression and survival of PCT. These data indicate that EEF1A2 may play a role in the induction or progression of plasma cell neoplasms in both mice and humans.

Materials and methods

Mice, lymphomas, tissue microarray, immunohistochemistry, and oligo microarray analyses of gene expression

The origins and characteristics of primary B cell lineage lymphomas from NFS.1V- congenic, B6.12-MYC, SJL-β2m<sup>−/−</sup>, IL6-TG, and BALB/c-gld/gld mice, and the techniques used for Eef1a2 transcriptional profiling of the lymphomas using oligonucleotide arrays, were detailed previously [23,26]. The expression of human EEF1A2 was studied from the dataset (GSM6477 in GEO) for samples of patients with MM, monoclonal gammopathy of undetermined significance (MGUS) and normal plasma cells using Affymetrix U133A microarrays. The differences in transcript levels between MGUS or MM and normal controls were examined by unpaired t-test with Welch’s correction. A tissue microarray of cutaneous biopsies of normal individuals or patients diagnosed with MM was purchased from Folio BioSciences (Columbus, OH). Immunohistochemical studies were detailed previously [27]. Mouse protocols were approved by Animal Care and Use Committees of the National Institute of Allergy and Infectious Diseases and the National Cancer Institute.

Cell lines, constructs, transfection, and antibodies

The MPC11 PCT cell line was obtained from American Type Culture Collection (ATCC). MOPC315, TEPC2372, TEPC4142, PCT-AP, RPC5, ABPC4 and ABPC20 PCT cell lines were provided by Dr. M. Potter (National Cancer Institute, National Institutes of Health [NIH], Bethesda, MD). Cells were maintained at 37°C 5% CO<sub>2</sub> in RPMI 1640 (Invitrogen Life Technologies) with 10% fetal bovine serum (FBS) (Hyclone), 10 mM sodium pyruvate, 1x glutamine, 1x non-essential amino acids, 0.5 μM β-mercaptoethanol and 0.1 M HEPES buffer. The human cell line, Raji, was purchased from ATCC. Cell lines ARK, MM-S1, XG-1 and XG-7 were from our laboratory and were gifts from Dr. Michael Kuehl (NCI, NIH).

Eef1a2 cDNA was cloned from the ABPC4 cell line and was inserted into the mammalian expression vector pcDNA3.2/V5 (Invitrogen) after sequencing. The sequencing results showed no mutations. pcDNA3.2/V5-CAT (chloramphenicol acetyltransferase) and pcDNA3.2/V5 (blank vector) were used for control and mock transfection, respectively. DNA (2 μg) of each construct was used to transfect 2×10<sup>6</sup> cells using transfection reagent solution V and program X-001 (Amaxa).

Specific anti-EEF1A2 polyclonal antibody was described previously [1]. Anti-EEF1A was from Upstate (Upstate Biotech, Charlotteville, VA), anti-β-actin was from Abcam, anti-V5 epitope was from Invitrogen, anti-STAT3, phosphorylated STAT3, PI3K, AKT and phosphorylated AKT were purchased from Cell Signaling Technology (location).

Stable knockdown of Eef1a2 expression

A 29mer-pRS-shRNA vector (Origene) was used to express shRNA. Sequences specific for mouse Eef1a2 knockdown: CTCATCTAGAAGTGGTGGGCATCGACA (shRNA-1); GTCAGCCCTACATCA AGAAGATCGGCTA (shRNA-2); ATCTCGGCGTGGCATGTGACACATGCT (shRNA-3); GTGACAAATGTGGGTTCTCAATGTGGAAGAT (shRNA-4); control sequences: TGCACCCTGACCTACGGCGTG-CAGTGC (shRNA-C). After transfection according to the methods mentioned above, 20 μg/ml puromycin was added into medium for selection. Single cell clones were maintained in medium with 10 μg/ml puromycin.

Western blot

Total cell lysates were prepared in RIPA lysis buffer (Pierce Chemical Co.) supplemented with protease inhibitor cocktail solution (Pierce Chemical Co.). Lysates were cleared by centrifugation at 13,000 g for 15 min at 4°C, and the protein content was determined using the BCA protein assay kit (Pierce Chemical Co.). 15 μg of protein per lane was separated on a NuPage 12% Bis-Tris gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Invitrogen). After blocking with a 5% skim milk solution, the blot was incubated with indicated antibodies. The primary antibodies were detected with horseradish peroxidase–conjugated secondary antibody (R&D system) and developed by Super Signal West pico detection kit (Pierce Chemical Co.) according to the manufacturer’s instructions.

Cell cycle and proliferation assay

1×10<sup>6</sup> cells expressing the Eef1a2 RNAi or control cells were harvested, washed with PBS, fixed with 70% ethanol overnight at minus 20°C, and treated with 10 μg/mL RNase (Roche). Cells were then stained with propidium iodide (PI) (5 μg/mL) and the cell cycle profile was determined using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Data are representative of three independent experiments and were analyzed with the FlowJo software (Tree Star, Inc., Ashland, OR).

For cell proliferation assays, the Click-iT EdU Flow Cytometry Assay Kit (Invitrogen), which is a BrdU alternative assay kit, was used according to the manufacturer’s instructions. Briefly, 1×10<sup>6</sup> cells/well were cultured overnight in 24-well plates. 10 μM EdU were then added to each well. 4 hours later, the cells were harvested and permeabilized immediately. After incubation with anti-EdU for 30 min, the cells were analyzed by flow cytometry.

Apoptosis assay

Apoptotic cells were differentiated from viable or necrotic cells by combined application of Annexin V-FITC and PI using the Vybrant Apoptosis Assay Kit #3 (Invitrogen). Briefly, cells were centrifuged and the cell pellet was suspended in 1x Annexin V binding buffer at a concentration of 1×10<sup>6</sup> cells/ml. Samples were incubated with 0.5 μg/ml Annexin V-FITC and 2 μg/mL PI for 10 min at room temperature and then were examined by flow cytometry. Data are representative of three independent experiments.

Quantitative Real-Time PCR (qPCR) and qPCR arrays

Total RNA was isolated using the RNasy mini kit coupled with DNase set (both from Qiagen). Reverse transcription was performed using 1 μg of RNA, 300 ng random hexamer primer (Invitrogen), and 200 units Superscript II (Invitrogen). The primers for qPCR were designed using Primer Express software (Applied Biosystems, Foster City, CA) and synthesized at Integrated DNA Technologies, Inc (Coralville, IA; Table S1). Each qPCR reaction was performed in a mix of 10 μl reaction mixture containing 2 ng of cDNA, 2×SYBR Green PCR Master
Mix (Applied Biosystems), and 0.3 μM of each forward and reverse primer on the ABI PRISM 7900HT sequence detector system (Applied Biosystems). All samples were tested in triplicate, and analyses were performed using SDS v2.2 software (Applied Biosystems) according to the manufacturer’s instruction. The comparative CT method (ΔΔCT) was used for quantification of gene expression. A single product for each primer pair was confirmed by gel electrophoresis and melt-curve analyses. The primers used for qPCR are listed in Table S1.

For qPCR array assays, cDNAs were applied to 384-well plates containing predetermined primer pairs representing various genes known to be cancer related and/or important for lymphoid cell development and function (Bar Harbor BioTechnology, Trenton, ME). PCR amplification was performed using regular SYBR-Green reagents (Applied Biosystems) and analyzed by a global pattern recognition algorithm as recently modified (http://array.lonza.com/apps/gpr/) [28].

The differentially expressed genes were classified by GO (Gene Ontology), and enrichment of significant genes was determined by Fisher’s exact test.

Results

Expression of Eef1a2 in primary mouse PCT

Eleven classes of mouse primary B cell lineage lymphomas were analyzed using oligonucleotide microarrays that queried over 11,000 genes. A t test was used to identify genes that distinguished each subset from all the others. These studies identified Eef1a2 as one of the genes highly expressed in PCT (Figure 1A), with mean transcript levels in PCT being around five-fold higher than in the other tumor classes. RT-PCR analyses of Eef1a2 expression in primary PCT, four other lymphoma subsets and normal spleen tissue showed that Eef1a2 transcripts were detected only in samples from PCT (data not shown). Immunohistochemical studies using an EEFA12-specific polyclonal antibody showed that EEF1A2 was expressed at high levels in the cytoplasm of PCT but not in MZL or other types of lymphomas (Figure 1B and data not shown). qPCR analyses of sorted mouse plasma cells and B220+ splenic B cells revealed that transcript levels for Eef1a2 were below the levels of detection in both populations (data not shown), an observation consistent with earlier studies showing that EEFA12 is normally expressed only in heart, muscle and brain. These studies established that among primary B cell lineage neoplasms of mice, EEFA12 is uniquely expressed at high levels in PCT, and that expression might be linked to pathogenesis because it is not expressed by normal plasma cells.

Expression of Eef1a2 in PCT cell lines

Recently, gene expression profiles were generated for six subtypes of pristane-induced mouse PCT. The groups included tumors induced by pristane alone as well as those from pristane-treated mice injected with acutely transforming retroviruses.
High-level expression of Ef1a2 was found in all of the subtypes (data not shown) indicating that expression of Ef1a2 in PCT was independent of the mode of PCT induction. We next analyzed EEF1A2 expression at the transcript and protein levels in eight PCT cell lines (Figure 1C, D). RT-PCR analyses showed that three lines (ABPC4, ABPC20 and PCT-AP) had substantially levels of Ef1a2 transcripts, while all eight expressed equivalent levels of transcripts for the closely related gene, Ef1a1, using \( \beta\)-actin transcript levels as a control (Figure 1C). Western blot analyses of EEF1A2 and EEF1A expression in the eight lines (Figure 1D) showed that EEF1A2 protein was present in protein extracts from the same tumors that were positive by RT-PCR. Comparative genomic hybridization (CGH) analyses of ABPC4 and ABPC20 cell lines showed no increase in copy number for the region of chromosome 2 where Ef1a2 resides (data not shown). These data indicated that high level expression of EEF1A2 by PCT was independent of their mode of induction and was not based on increased copy number, at least in the cases examined.

Expression of EEF1A2 in purified plasma cells from normal bone marrow, from individuals with MGUS or primary MM cells and from MM cell lines

We next used published gene expression profiling (GSM6477 in GEO) to study EEF1A2 transcript levels in sort-purified CD138+ bone marrow plasma cells from 15 normal controls, 22 patients with MGUS and 125 primary cases of MM (Figure 2A). While EEF1A2 transcript levels were very low in normal controls, the levels in plasma cells from individuals with MGUS, non-transformed precursors to probably all cases of MM, were significantly higher (p = 0.0001). Furthermore, the levels in MM were also significantly higher than normal controls (p < 0.0001) but were not significantly higher than for MGUS. High levels of EEF1A2 transcripts and protein were confirmed for three human...
MM cell lines by qPCR and western blot analyses, respectively (Figure 2B, 2C).

We extended these analyses of MM by immunohistochemical studies using a tissue microarray containing bone marrow biopsies from 20 normal controls and 20 patients with MM. The results showed that EEF1A2 protein was detected at background to low levels in biopsies from normal controls and five cases of MM but at high levels in 15% of primary MM (Figure 2D, 2E). This suggests that levels of EEF1A2 expression in primary MM tumor cells may be determined post-transcriptionally as well as translationally and clearly deserves further study. We conclude that expression of EEF1A2 is progressively upregulated during the progression of normal plasma cells to MGUS and MGUS to MM.

Transient and stable silencing of Eef1a2 in PCT cell lines

To understand the consequences of high-level expression of EEF1A2 in PCT, we transiently transfected the PCT-AP PCT cell line with 4 different shRNAs directed at Eef1a2 as well as a control shRNA and quantified Eef1a2 transcript levels by qPCR. As shown in Figure S1A, the expression of Eef1a2 was markedly downregulated in cells transfected with shRNA-3 and shRNA-4 while the other two shRNAs were much less effective and the control, shRNA-C, had no effect. We also generated stable transfectants of shRNA-3 and the control shRNA-C in the ABPC4 PCT cell line following selection with puromycin. The Eef1a2 knockdown cell line had barely detectable levels of Eef1a2 transcripts (Figure S1B, left panel) and EEF1A2 protein (Figure S1B, right panel), but normal levels of Eef1a2 transcripts and relatively normal levels of EEF1A protein (data not shown). This indicated that Eef1a2 transcripts were specifically silenced by the targeting shRNA-3 in ABPC4.

Knockdown of Eef1a2 inhibits cell growth and cell proliferation

It was reported that human EEF1A2 promoted cell growth and proliferation in human ovarian cancer. To examine the relationship between EEF1A2 expression and PCT growth rates, we compared cell numbers of ABPC4 expressing control shRNA-C or Eef1a2 shRNA-3 during four days in culture (Figure 3A). The

Figure 3. Knockdown of Eef1a2 inhibits cell growth and cell proliferation. (A) ABPC4 cell numbers stably expressing Eef1a2 shRNA-3 and control shRNA-C cells were determined during four days in culture. (B, C) Overexpression of EEF1A2 protein in the MPC11 cell line (B). Cell numbers were determined during culture after transfection (C). (D) The frequency of EdU-positive MPC11 cells in transiently transfected Eef1a2 and control plasmid was analyzed by flow cytometry (left). The statistic bars show on the right. (E) The frequency of EdU-positive PCT-AP cells in transiently transfected Eef1a2 shRNAs, and control shRNA expressing PCT-AP cells was analyzed by flow cytometry.

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growth of cells expressing shRNA-3 was significantly reduced after 72 h and 96 h, with cell numbers in these cultures being only half that of untransfected cells or cells transfected with shRNA-C.

The observed changes in cell number could be due to reduced cell cycle progression, increased apoptosis or both. To examine these possibilities, we first evaluated cells for EdU uptake after 4 h in culture using stably transfected cell lines. These studies showed that the frequency of EdU-positive cells in the Eef1a2 shRNA-3 expressing cells was about half that of normal cells or cells expressing the inactive shRNA (Figure S2A). To extend this observation, we analyzed cell proliferation in transiently transfected cells from a second PCT cell line, PCT-AP. The results shown in Figure 3D indicated that knockdown of Eef1a2 was associated with inhibition of PCT-AP proliferation, and that the inhibitory effect was related to the knockdown efficacy of the individual shRNAs (Figure 3E). Taken together, these results indicated that EEF1A2 promotes cell growth in the two PCT cell lines examined.

These observations prompted us to see if the growth of a PCT cell line, MPC11, that does not express EEF1A2 (Figure 1C, D), would be enhanced by introducing an EEF1A2 expression vector. To this end, we transiently expressed mouse Eef1a2 or CAT, as a control, in MPC11 under the control of the CMV promoter. The Eef1a2 and CAT constructs were tagged at the carboxy termini with the V5 epitope to facilitate detection by western blotting. Equivalent levels of EEF1A2 and CAT protein were expressed in the transfected cells (Figure 3B). The growth of EEF1A2 expressing cells was only modestly increased over those expressing CAT, best seen at 72 hr [P<0.05] (Figure 3C). Edu binding assay also showed increased proliferation (Figure 3D). Together, our results demonstrated that expression of EEF1A2 enhanced proliferation of PCT cell lines.

**Eef1a2 knockdown delays cell cycle entry**

Next, we asked if the effect of EEF1A2 on cell growth might also be related to altered cell cycle regulation. Untransfected ABPC4 cells and cells with the active and inactive shRNAs were stained with PI and examined by flow cytometry (Figure 4A). Eef1a2 knockdown cells had significantly increased percentages of cells in G1 and significantly decreased proportions of cells in both the S and G2/M stages of the cell cycle (Figure 4A), indicating that down regulation of Eef1a2 delayed G1 to S progression to a limited but significant extent. Moreover, the absence of a sub-G1 accumulation showed that apoptosis was not significantly increased after knockdown of Eef1a2 expression with cells grown in serum-containing medium.

**Serum-free conditions enhance apoptosis in Eef1a2 knockdown cells**

Pro- or anti-apoptotic effects of some genes become evident only under conditions of cellular stress. This prompted us to determine if depletion of EEF1A2 in ABPC4 might affect the viability of cells...
Knockdown of Eef1a2 expression alters expression of many genes involved in proliferation and signaling

Apart from its canonical function, it is known that EEF1A2 can activate the AKT signaling pathway by binding directly to PI4K2B [12,31,32]. To gain further insight into the consequences of Eef1a2 knockdown in PCT, we performed gene expression profiling using a 304 well qPCR array enriched for genes known to be involved in hematopoietic neoplasms. Genes for which expression was substantively altered in the stable Eef1a2 knockdown cell line are listed in Figure 5A in relation to their functional categorization by gene ontology and analyses of enrichment within each category. Significant enrichment was observed for genes involved in proliferation (p = 0.016), in keeping with the data presented above, and signaling (p = 0.026). Among the genes involved in signaling, there was significant enrichment specifically in genes involved in the JAK/STAT1 signaling pathway (p = 0.010) (Table S2). These results showed that EEF1A2 might be involved in the regulation of JAK/STAT signaling as well as the AKT pathway as reported previously [12,20,32].

To examine these possibilities, we treated the Eef1a2 knockdown cell line and control RNAi cell line with IL-6, and examined them for expression of STAT3, pSTAT3, AKT, pAKT and -actin (Figure 5B). We noted that phosphorylated STAT3 was readily detectable after 15 min in control RNAi cells, but reached similar levels only after 30 min in Eef1a2 RNAi cells. pAKT was detectable in both cell lines at very low levels at 15 min after stimulation, but increased substantially at 30 min only in control cells. These results indicated that knockdown of Eef1a2 expression delayed or impaired IL-6-induced activation of both the STAT3 and AKT signaling pathways. Importantly, the protein level of PIK3CG, which is upstream of AKT, was significantly decreased in the Eef1a2 RNAi cell line at all time points. Furthermore, transcript levels of Pik3cg were decreased in Eef1a2 RNAi cells (Table 1) indicating that control of PIK3CG protein expression was determined at the transcriptional level.

Among the genes with significantly altered expression in Eef1a2 RNAi cells (Table 1) were those involved in tumor invasion (Tgfb2, Mmp13, Ibg), proliferation (Pik3cg, Fosb, Fos, Mapk1, Pyk2, Wnt1, Cdk6, Ccnd2), survival (Ijg2020b, Tufjsf13b, Be2, Be2l21l1), and cytokine and interferon signaling (Jak2, Stat1, Stat2, Stat3, Ifi2, Ifi8, Socs3). Most interestingly, a number of the genes contain SH2 or SH3 domains - JAK2, STAT1, STAT2, STAT3, SOCS3, FYN, LYN, and PIK3CG - supporting the suggestion from previous studies that EEF1A2 may directly or indirectly interact with proteins containing SH2 or SH3 domains [33,34]. Furthermore, increased levels of transcripts for Be2 and Be2l21l1 may affect the limited degree of apoptosis associated with knockdown of Eef1a2 expression.

To further validate our qPCR array data, we analyzed the expression of Tgfb2, Be2, Cdk6, Mmp13, Pik3cg and Stat3, genes that are significantly regulated in ABPC4 cells with a stable knockdown of Eef1a2 (Table 1) or using PCT-AP cells transiently transfected with knockdown and control shRNAs (Figure 5C). After transfection for 48 hours, all six genes showed changes in expression that were consistent with the data obtained from stably transfected cells (Table 1). In addition, some of the regulatory effects could be related to the knockdown efficacy of the individual shRNAs (Figure S1A). We conclude that EEF1A2 affects the expression of a number of signaling pathways.

Discussion

In tissues of normal animals, EEF1A2 is expressed only in heart, brain and muscle. The data presented here indicate that EEF1A2 is aberrantly expressed at high levels in some plasma cell neoplasms of mice and humans. This is the first example of EEF1A2 being expressed in a mouse tumor, although high-level expression has been documented in a variety of human tumors belonging to different cell lineages [1,2,3,4,5,6,7]. In contrast to earlier studies suggesting that increased expression of EEF1A2 is associated with terminal differentiation [35], normal mouse and human plasma cells were found to be EEF1A2-negative. Heightened expression cannot be tied uniquely to transformation within the B cell lineage as other subsets of mouse B cell tumors did not express Eef1a2. Furthermore, additional studies, although limited, suggest that heightened expression in these tumors is not due to mutation or amplification of the gene. Previous studies of ovarian tumors that aberrantly express EEF1A2 at high levels also ruled out contributions of mutation or changes in the methylation status of the gene, and showed that levels of expression did not correlate with gene amplification [36]. Interestingly, heightened levels of EEF1A2 transcripts in MM related most closely to a subset of cases that lack primary IgH translocations and overexpress D-type cyclins. Features of this MM subset that might drive EEF1A2 expression remain to be determined.

The observations that Eef1a2 transcripts are expressed at increased levels in the majority of primary PCT but in a smaller proportion of primary MM indicate that the mechanisms governing aberrant expression and possible contributions to transformation of plasma cells are likely to differ between the species. Whether these differences are cell intrinsic or species-dependent remain to be determined.

Our studies of mouse PCT suggest that heightened expression of EEF1A2 might contribute to transformation by promoting cell cycle progression and inhibiting apoptosis. Support for this view comes from prior studies of non-PCT cell lines overexpressing EEF1A2 that exhibited enhanced cell growth [20] and resistance to apoptosis [14,21]. Our studies of PCT cell lines suggest that activation of STAT3 and AKT may contribute to inhibition of apoptosis.

Although several mouse models have emerged as useful platforms for mechanistic and therapeutic studies of alterations in signaling pathways found in human MM (e.g., IL-6, Abl, and c-Myc), none of the models faithfully recapitulates all features of human MM. We screened 11 types of mouse lymphoma by microarray and found Eef1a2 as a candidate cancer gene that was expressed at high levels only in PCT. None of the other classes of B cell lineage tumors expressed Eef1a2 at high levels, including anaplastic and plasmablastic PCT, which have a number of histological and molecular similarities to plasmacytic PCT [22,37]. Since Eef1a2 is not expressed in normal plasma cells, our findings suggest that inappropriate expression of EEF1A2 in B cell differentiation may contribute to the induction, progression or survival of a high proportion of primary PCT. Eef1a2 was expressed at high levels by PCT from mice of four different genetic
backgrounds that had been subjected to a variety of PCT induction protocols. We also found EEF1A2 was highly expressed at the protein level in 15% of primary cases of MM as well as a number of MM cell lines. Interestingly, levels of EEF1A2 transcripts were increased to a lesser extent in plasma cells of individuals with MGUS, a consistent precursor to MM [38], than in primary MM. This suggests that activation occurred during the transition of normal plasma cells to MGUS with the levels in cases of primary MM not being significantly higher than in MGUS. These observations indicate that deregulated expression of

Figure 5. Functional changes after Eef1a2 knockdown. (A) Functional classification of differentially expressed genes by Eef1a2 knockdown cells. (B) Downregulation of Eef1a2 impaired IL-6-induced AKT and STAT3 phosphorylation. Eef1a2 shRNA and control cells were treated with 100 ng/ml recombinant IL-6 and protein samples were prepared 15 and 30 min later. Western blot analyses were performed using the indicated antibodies. (C) qPCR analyses of gene expression levels in transiently transfected shRNAs in PCT-AP cell line with four plasmids expressing specifically targeting Eef1a2 (shRNA-1,2,3,4) and a control plasmid (shRNA-C). Error bar = ± S.E.

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Table 1. Genes expressed at significantly increased or decreased levels in Eef1a2-knockdown cell line compared to control cell line.

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<th>Gene Name</th>
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**Table S1**: Specific knockdown Eef1a2 expression in PCT cell lines. (A) qPCR analyses of Eef1a1 and Eef1a2 expression levels in transiently transfected PCT-AP cell line with four plasmids specifically targeting Eef1a2 (shRNA-1,2,3,4) and a control plasmid (shRNA-C). (B) Eef1a1 and Eef1a2 transcripts (left) and EEF1A2
protein (right) levels were analyzed by qPCR and western blotting, respectively, in stably transfected ABPC4 cells with a plasmid expressing shRNA-3 specifically targeting Eef1a2 and a plasmid expressing control shRNA-C. Error bar = ± S.E. **p<0.01. Found at: doi:10.1371/journal.pone.0010755.s001 (0.02 MB PDF)

Figure S2 (A) The frequency of EdU-positive cells in the Eef1a2 shRNA-3 expressing, control shRNA-C expressing cells and control cells were analyzed by flow cytometry. Error bar = ± S.E. **p<0.01. (B) Apoptotic cells were analyzed by flow cytometry in cells transiently transfected with Eef1a2 shRNAs and shRNA-C after culturing in serum-free medium for 48 hours. Found at: doi:10.1371/journal.pone.0010755.s002 (0.05 MB PDF)

Table S1 Primers used for qPCR.

Table S2 Signaling classification for differentially expressed genes by Eef1a2 knock-down.

References


Found at: doi:10.1371/journal.pone.0010755.s004 (0.10 MB PDF)

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Author Contributions

Conceived and designed the experiments: ZL CMA HCM. Performed the experiments: ZL CFQ HJN ALK CMA HCM. Analyzed the data: ZL CFQ DMS AZ HJN ALK CMA HCM. Contributed reagents/materials/analysis tools: ZL CMA HCM. Wrote the paper: ZL HCM.