Overexpression of c-maf is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma

Elaine M. Hurt,[1] Adrian Wiestner,[1,2] Andreas Rosenwald,[1] A.L. Shaffer,[1] Elias Campo,[4] Tom Grogan,[5] P. Leif Bergsagel,[6] W. Michael Kuehl,[3] and Louis M. Staudt[1,*]

[1]Metabolism Branch, Center for Cancer Research, National Cancer Institute
[2]Hematology Branch, National Heart Lung and Blood Institute
[3]Genetics Branch, Center for Cancer Research, National Cancer Institute
National Institutes of Health, Bethesda, Maryland 20892
[4]University of Barcelona, 08036 Barcelona, Spain
[5]University of Arizona Medical School, Tucson, Arizona 85724
[6]Weill School of Medicine, New York, New York 10021
*Correspondence: lstaudt@mail.nih.gov

Summary

The oncogene c-maf is translocated in ∼5%–10% of multiple myelomas. Unexpectedly, we observed *c-maf* expression in myeloma cell lines lacking *c-maf* translocations and in 50% of multiple myeloma bone marrow samples. By gene expression profiling, we identified three *c-maf* target genes: *cyclin D2*, *integrin β7*, and *CCR1*. c-maf transactivated the *cyclin D2* promoter and enhanced myeloma proliferation, whereas dominant inhibition of c-maf blocked tumor formation in immunodeficient mice. c-maf-driven expression of integrin β7 enhanced myeloma adhesion to bone marrow stroma and increased production of VEGF. We propose that c-maf transforms plasma cells by stimulating cell cycle progression and by altering bone marrow stromal interactions. The frequent overexpression of c-maf in myeloma makes it an attractive target for therapeutic intervention.

Introduction

Multiple myeloma, an incurable malignancy of the plasma cell, accounts for roughly 20% of all hematological malignancies. Clues to the pathogenesis of myeloma are coming from two general approaches, one that focuses on recurrent translocations and mutations of oncogenes and the other that emphasizes the interplay between the malignant plasma cells and the bone marrow microenvironment (Kuehl and Bergsagel, 2002). Four recurrent translocations involving the immunoglobulin heavy chain (IgH) locus are likely to be primary oncogenic events based on their occurrence in a premalignant lesion termed monoclonal gammopathy of undetermined significance (MGUS) (Fonseca et al., 2002), and their use of illegitimate IgH switch recombination (Kuehl and Bergsagel, 2002). The t(11;14) and t(6;14) translocations involve the *cyclin D1* and *cyclin D3* genes, respectively, and presumably disturb the physiological exit of plasma cells from the cell cycle. The t(4;14) translocation deregulates both the *FGFR3* and *MMSET* genes and the t(14;16) translocation deregulates the *c-maf* gene, but the functional consequences of these translocations are unknown.

Recent work has also demonstrated that interaction of myeloma cells with bone marrow stroma provides the cells with growth and survival factors such as IL-6 and VEGF and promotes chemotherapeutic drug resistance (Dalton, 2002; Hideshima and Anderson, 2002). Although a number of adhesion molecules are present on the surface of multiple myeloma cells, it is not clear which are critical for binding to stromal cells (Cook et al., 1997).

In the present study, we investigated how the oncogene *c-maf* contributes to the pathogenesis of multiple myeloma. c-maf is a member of the basic-leucine zipper family of transcription factors that has been shown to be important for IL-4 gene expression by T-helper-2 cells (Ho et al., 1996; Kim et al., 1999a), and formation of the lens (Kim et al., 1999b). To date, the molecular targets of c-maf transactivation that are important for malignant transformation in multiple myeloma have not been elucidated. In the present study, we used gene expression profiling to identify cyclin D2 and integrin β7 as c-maf target genes in multiple myeloma, and demonstrate that c-maf increases myeloma proliferation and adhesion to bone marrow stroma.

SIGNIFICANCE

Recurrent translocations have been identified in multiple myeloma, but their functional significance is largely unknown. Unexpectedly, we found that roughly 50% of myelomas overexpress the oncogene c-maf, although c-maf translocations occur in only 5%–10% of cases. c-maf increased the expression of cyclin D2 and integrin β7, leading to enhanced proliferation and increased adhesion to bone marrow stroma. Inhibition of c-maf function potently blocked tumor formation in immunodeficient mice, suggesting that c-maf is an attractive therapeutic target for roughly one half of myeloma patients.

COPYRIGHT © 2004 CELL PRESS

Notably, we found c-maf to be overexpressed and functional in roughly one half of myelomas, leading us to propose that c-maf is an attractive therapeutic target in this malignancy.

Results

Identification of c-maf target genes

To gain insight into the mechanism of action of multiple myeloma oncogenes, we used DNA microarrays to profile gene expression in a panel of 28 multiple myeloma cell lines that have been extensively characterized with respect to translocations, gene copy number changes, and oncogene mutations (Bergsagel et al., 1996). Cell lines with translocations of the *cyclin D1* and *MMSET/FGFR3* genes expressed these genes more highly than cell lines lacking these translocations (data not shown). Unexpectedly, *c-maf* was not only expressed in the 6 cell lines with *c-maf* translocations, but also in cell lines lacking this translocation (data not shown). To confirm and extend this observation, we measured *c-maf* mRNA levels using a quantitative RT-PCR assay in the 28 cell lines (Figure 1A). We found that 13 (46%) expressed high levels, 3 (11%) expressed low levels, and the remainder had no detectable *c-maf* expression. Although c-maf expression varied over a wide range, some cell lines lacking a *c-maf* translocation expressed *c-maf* as highly as some with a *c-maf* translocation. It is interesting to note that c-maf is overexpressed in all cell lines with an *MMSET/FFGR3* translocation and one cell line with a *cyclin D1* translocation. We also detected frequent *c-maf* expression in primary multiple myeloma cells purified from patient bone marrow aspirates (13/26 samples; 50%) (Figure 1A), which was surprising since *c-maf* translocations occur in only 5%–10% of myeloma cases (Kuehl and Bergsagel, 2002). *c-maf* mRNA was undetectable in normal plasma cells purified from bone marrow aspirates, demonstrating that *c-maf* overexpression in multiple myeloma is associated with the malignant process (Figure 1A).

To discover potential c-maf target genes, we searched the myeloma cell line gene expression dataset for genes that were differentially expressed between c-maf-expressing and -nonexpressing cell lines. The three genes that satisfied the selection criteria ($p < 0.001$; t test) were *integrin* β*7*, *cyclin D2*, and *C-C chemokine receptor-1* (*CCR1*) (Figure 1B). Even cell lines with relatively low c-maf expression (e.g., XG-2) expressed these putative c-maf target genes at higher levels than c-maf-negative cell lines (Figure 1B). We confirmed the coregulation of *c-maf* and *integrin* β*7* in the myeloma cell lines by quantitative RT-PCR, and also observed this coregulation in the primary multiple myeloma samples (Figure 1A). Although a few myeloma cell lines lacking *c-maf* expression had low levels of *integrin* β*7* mRNA, patient samples and normal bone marrow plasma cells lacking *c-maf* expression did not express *integrin* β*7* detectably (Figure 1A).

To directly search for c-maf target genes, we manipulated the activity of c-maf in myeloma cell lines and profiled the resultant changes in gene expression. Using retroviral transduction, we overexpressed c-maf in two cell lines that have no endogenous c-maf expression (KMS12 and L363). Additionally, we created a dominant negative form of c-maf, termed Ac-maf, by replacing the basic DNA binding region of c-maf with an acidic region while retaining the leucine zipper (Olive et al., 1997). We used retroviruses to transduce Ac-maf into two cell lines that express c-maf without a translocation (H929 and LP-1), and into one cell line that expresses c-maf as a consequence of a t(14;16) translocation (JJN3) (Chesi et al., 1998). The three genes that were consistently upregulated by c-maf overexpression and downregulated by Ac-maf were again *integrin* β*7*, *cyclin D2*, and *CCR1*, thus confirming that these genes are under the control of c-maf (Figure 2A). No genes were consistently repressed by c-maf or induced by Ac-maf (data not shown). Ac-maf had no effect on gene expression in two cell lines that do not express c-maf (L363 and KMS12; Figure 2A), demonstrating the specificity of this dominant negative protein for c-maf. As expected from the changes in mRNA abundance, overexpression of c-maf increased cyclin D2 and integrin β*7* protein levels (Figures 2C and 2D) and Ac-maf decreased integrin β*7* protein levels in c-maf-positive cells (Figure 2D).

To confirm that these target genes are downstream of c-maf action, we used siRNA to reduce expression of c-maf mRNA in H929 cells. Quantitative RT-PCR confirmed that *c-maf*, *integrin* β*7*, *cyclin D2*, and *C-C chemokine receptor-1* (*CCR1*) mRNAs were knocked down by c-maf siRNA in H929 cells (Figure 2B). c-maf siRNA transduction also decreased surface expression of integrin β*7* (Figure 2D).

The *cyclin D2* promoter contains a potential c-maf binding site that matches the consensus c-maf binding (MARE) motif and is well conserved in the human and mouse orthologs (Figure 2C). c-maf was able to directly transactivate a cyclin D2 promoter construct in transient transfection assays (Figure 2C). A 7 bp mutation that disrupts the key c-maf contact nucleotides of the MARE motif abrogated the ability of c-maf to transactivate the *cyclin D2* promoter (Figure 2C). These observations provide evidence that *cyclin D2* is a direct target of c-maf transactivation.

c-maf augments myeloma proliferation and tumor formation

Since c-maf increased cyclin D2 protein levels (Figure 2C), we investigated whether c-maf affected cell cycle progression. Retroviral transduction of c-maf into myeloma cells lacking endogenous c-maf expression caused these already cycling cells to increase their division and DNA synthesis (Figure 3A). We observed no evidence of increased cell death induced by dominant negative c-maf (data not shown). This finding is consistent with the idea that the c-maf-driven increase in cyclin D2 levels augments entry of cells into the cell cycle. Conversely, in most (6/7) c-maf expressing myeloma cell lines, introduction of Ac-maf decreased their division, including cell lines with and without a c-maf translocation (Figure 3B). As expected, Ac-maf had no effect on the proliferation of two c-maf negative cell lines (Figure 3B), again confirming the specificity of this dominant negative protein for c-maf.

Given the effect of c-maf on proliferation in vitro, we tested whether tumor formation in vivo by c-maf-expressing myelomas was dependent on c-maf (Figure 4). NOD-SCID immunodeficient mice were injected intraperitoneally with LP-1 cells that had been transduced with a retrovirus expressing dominant negative Ac-maf or with a control retrovirus. Tumor formation was assessed by measuring human immunoglobulin in the blood and by the appearance of visible abdominal masses (Figures 4A and 4B). All four mice injected with the control LP-1 cells developed tumors within 26–36 days and had rapidly rising titers of human immunoglobulin in their blood (Figures 4A and 4B). By contrast, the mice injected with dominant negative Ac-

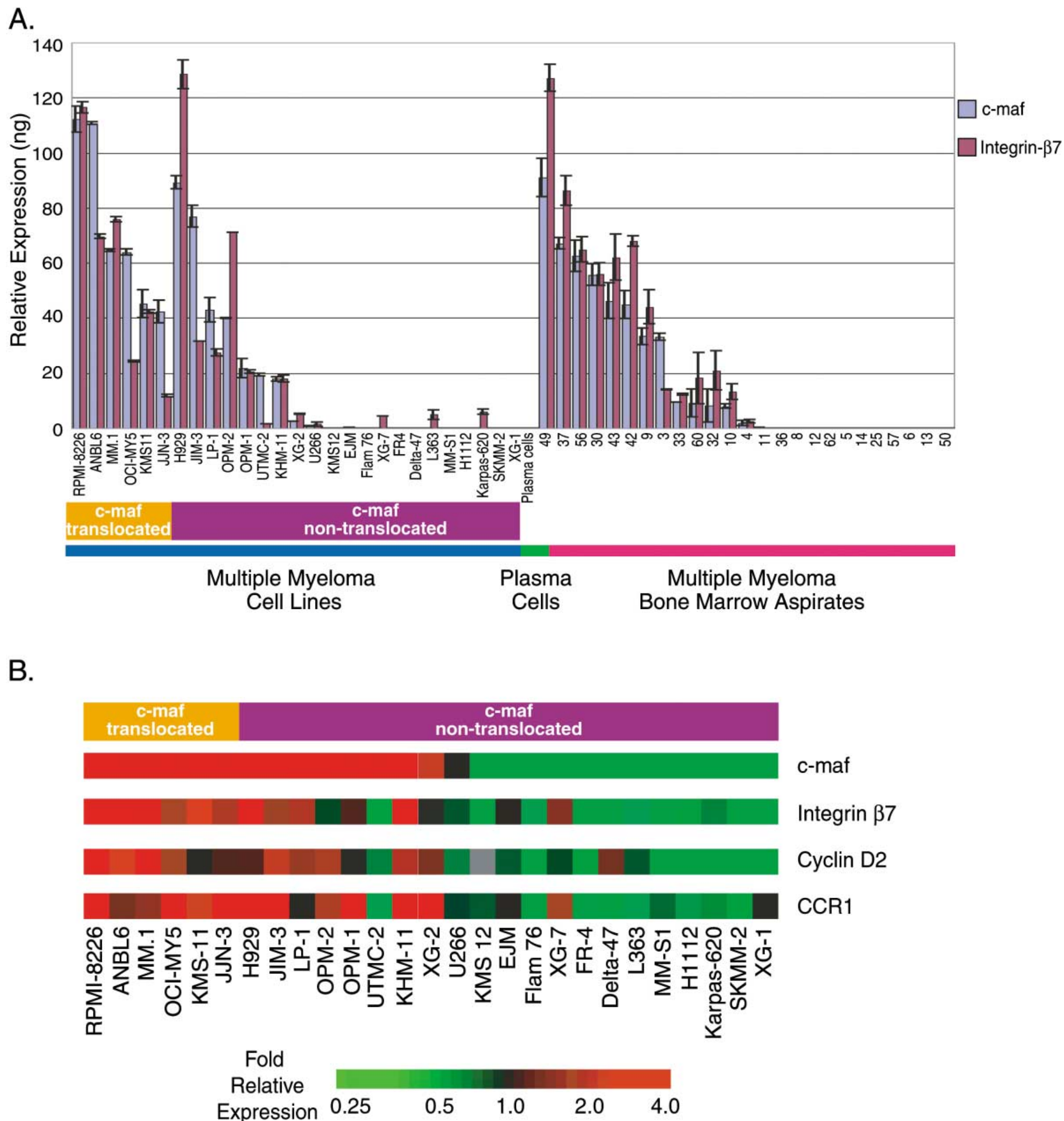

Figure 1. Frequent overexpression of c-maf and coregulated genes in multiple myeloma cell lines and patient samples

A: Expression levels of *c-maf* and *integrin β7* in myeloma cell lines, myeloma patient samples, and normal bone marrow plasma cells as determined by a quantitative reverse transcription polymerase chain reaction assay.

B: Genes coregulated with c-maf in multiple myeloma cell lines. Gene expression was assessed by quantitative RT-PCR (*c-maf*) or DNA microarray measurements (*integrin β7*, *cyclin D2*, *CCR1*). Relative gene expression levels are depicted according to the color scale shown in which shades of red indicate higher expression and shades of green indicate lower expression. Gray indicates missing data.

maf LP-1 cells either did not form tumors (2/4) or formed tumors at a later time point than the mice with control LP-1 cells (Figure 4B). Interestingly, the two tumors that arose in mice receiving Ac-maf LP-1 cells had little or no expression of Ac-maf protein at the time of sacrifice, unlike the cells that were originally injected in these animals (Figure 4C). The emergence of these escape tumors, together with the fact that Ac-maf prevented tumor formation altogether in 2/4 mice, provides evidence that inhibition of c-maf function blocks the in vivo proliferation of c-maf-expressing myelomas.

c-maf increases myeloma adhesion to bone marrow stroma

Since the interaction of the bone marrow stroma with multiple myeloma cells is an important component of the pathophysiology of this malignancy (Anderson, 2001; Shain et al., 2000), we asked whether the upregulation of integrin β7 by c-maf altered the adhesion properties of myeloma cells. Integrin β7 can heterodimerize with either integrin α4 or integrin αE. Integrin α4β7 binds to MadCAM-1 and integrin αEβ7 binds to E-cadherin (Hynes, 2002). E-cadherin has been detected on the surface of

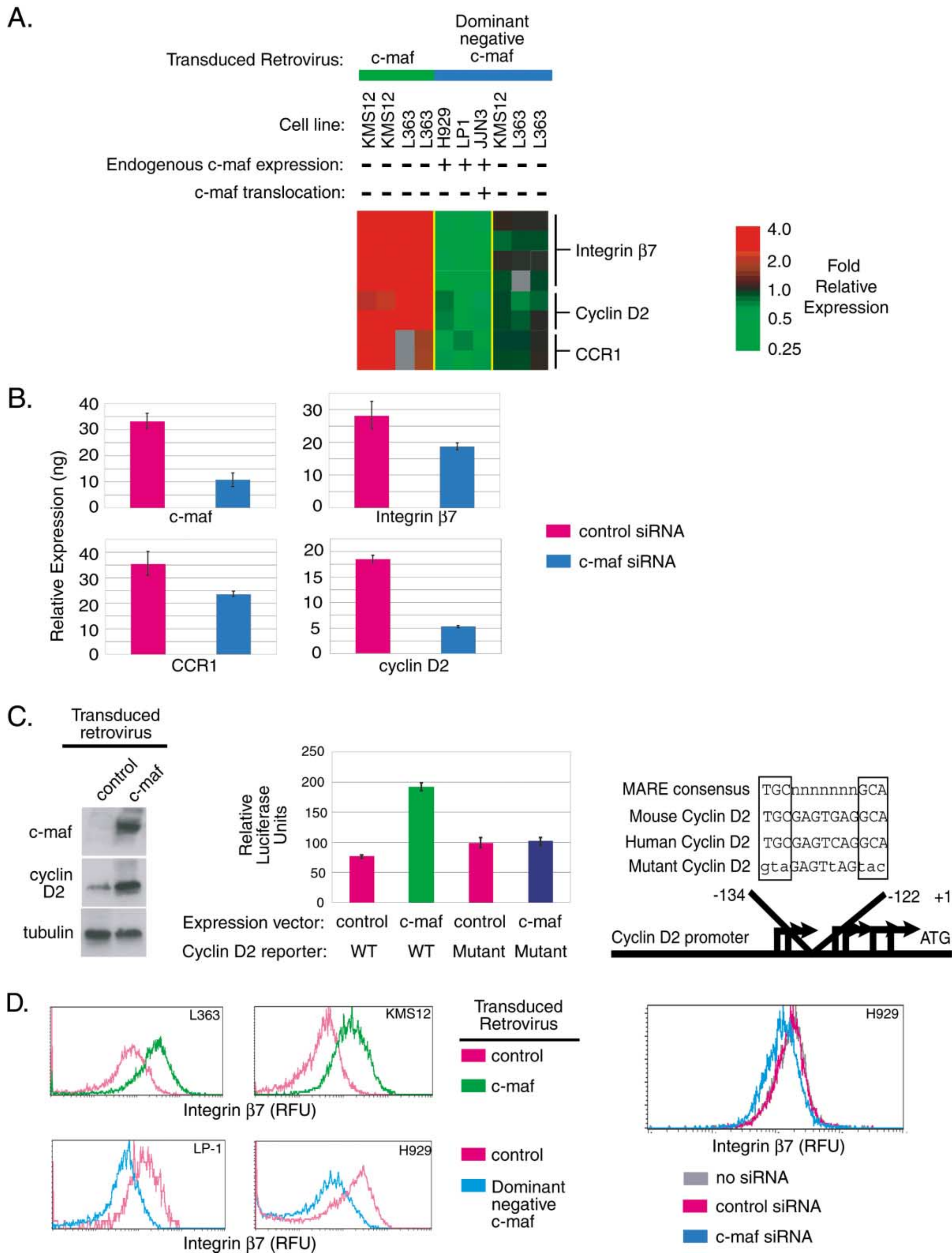

Figure 2. c-maf regulates the expression of *integrin β7*, *cyclin D2*, and *CCR1* and activates the cyclin D2 promoter

A: c-maf function in myeloma cell lines was manipulated by retroviral transduction of c-maf or a dominant negative form of c-maf. Gene expression changes are depicted according to the color scale in which red indicates an increase, green indicates a decrease, and black indicates no change. Gray indicates missing data. Multiple microarray elements are shown for each target gene.

B: Quantitative RT-PCR assay for c-maf, integrin β7, cyclin D2, and C-C chemokine receptor-1 mRNA of H929 cells 72 hr after being transduced with a c-maf siRNA oligonucleotide.

C: c-maf overexpression increases cyclin D2 protein levels. Western blot analysis of L363 cells that have been transduced with a retrovirus expressing c-maf or a control retrovirus is shown. β-tubulin serves as a loading control. Also shown is the regulation of the cyclin D2 promoter by c-maf. KMS12 myeloma cells were transfected with a c-maf expression construct or a control vector together with a luciferase reporter construct driven by either a wild-type or mutant

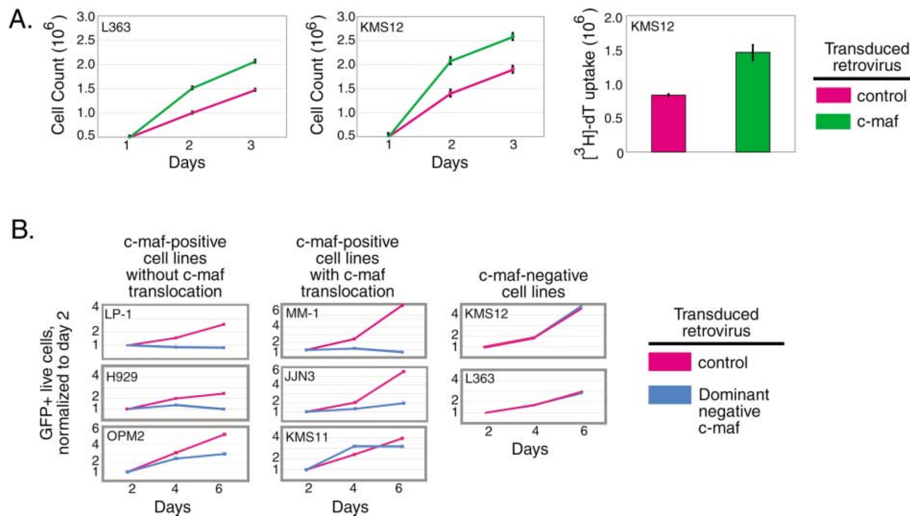

Figure 3. c-maf overexpression promotes proliferation in myeloma cell lines while dominant negative inhibition of c-maf retards proliferation

A: Overexpression of c-maf enhances proliferation and DNA synthesis. Growth curves of two myeloma cell lines lacking endogenous c-maf expression (L363 and KMS12) that have been transduced with a retrovirus expressing c-maf or a control retrovirus. An average of two experiments preformed in triplicate is shown with error bars representing the standard deviation. Also shown is the incorporation of H^3-thymidine into DNA in KMS12 cells transduced with a c-maf expressing retrovirus or a control retrovirus.

B: Inhibition of c-maf reduces the proliferation of c-maf-expressing cell myeloma cells. Retroviruses expressing a dominant negative form of c-maf and GFP from a bicistronic mRNA, or GFP alone were used to infect the indicated cell lines. The number of GFP+ live cells in the cultures over time were determined relative to the number at day 2 following retroviral transduction.

bone marrow stromal cells (Turel and Rao, 1998). Two cell lines that express endogenous c-maf (H929 and LP-1) adhered to E-cadherin-coated plates, whereas two cell lines lacking c-maf expression (L363 and KMS12) did not bind to E-cadherin-coated plates (Figure 5A). Retroviral transduction of c-maf into these latter cell lines conferred binding to E-cadherin-coated plates (Figure 5A).

Next we investigated the role of c-maf in the interaction of myeloma cells with bone marrow stromal cells (Figure 5B). Cell lines expressing c-maf were able to adhere to the stroma, presumably through the concerted action of several adhesion molecules (Anderson, 2001; Shain et al., 2000). Preincubation of the myeloma cells with an anti-integrin β7 antibody resulted in a 40%–72% decrease in adherence to the stroma (Figure 5B). Preincubation of the stroma with an E-cadherin antibody blocked adherence of myeloma cells to a similar degree (Figure 5B). Myeloma cell lines lacking c-maf expression were able to adhere to stroma, but transduction of these cells with the c-maf retrovirus increased adherence 2.5- to 3.5-fold (Figure 5C). Antibodies to integrin β7 or E-cadherin completely blocked the increased adherence caused by c-maf (Figure 5C). We developed a competitive binding assay in which fluorescently labeled, c-maf-negative cells were allowed to adhere to stroma in the presence of increasing numbers of unlabeled cells; c-maf-expressing cells were more efficient competitors than cells lacking c-maf (Figure 5D). These data suggest that c-maf enhances the adhesion of myeloma cells to bone marrow stroma through interactions between integrin αEβ7 and E-cadherin.

Interaction of myeloma cells with bone marrow stroma induces secretion of vascular endothelial growth factor (VEGF), which may act in an autocrine and paracrine fashion to promote myeloma proliferation and survival, and may induce neovascularization (Bellamy et al., 1999; Dankbar et al., 2000; Gupta et al., 2001; Podar et al., 2001, 2002). We therefore investigated whether the enhanced stromal interaction caused by c-maf results in increased VEGF secretion (Figure 5E). Myeloma cells and stroma cells alone did not secrete appreciable quantities of VEGF, but cocultures of stroma with myeloma cells lacking c-maf expression produced more VEGF (2.3-fold). Even greater VEGF secretion (5.5-fold) occurred in cocultures of stroma with c-maf-expressing myeloma cells. Again, this augmented VEGF production was dependent upon integrin αEβ7 interactions with E-cadherin since antibodies to these two adhesion proteins reduced VEGF secretion to the levels observed using c-maf nonexpressing myeloma cells. These data suggest that c-maf alters the nature of the myeloma/stromal cell interaction and that interaction leads to an increase in VEGF secretion.

Discussion

The present analysis of c-maf transcriptional targets in multiple myeloma suggests that c-maf may promote malignant transformation of plasma cells by at least two distinct mechanisms (Figure 6). One mechanism involves the enhanced proliferation of myeloma cells in a cell autonomous fashion. Plasmacytic differentiation is accompanied by upregulation of the cyclin-dependent kinase inhibitor, p18 (Tourigny et al., 2002). Myelomas apparently must circumvent this physiological cell cycle arrest since two recurrent translocations in myeloma involve the *cyclin D1* and *cyclin D3* genes, and homozygous deletions of the *p18* locus can occur (Kulkarni et al., 2002). Our results suggest that the transcriptional activation of *cyclin D2* by c-maf

cyclin D2 promoter. Luciferase activity is indicated as an average of triplicate assays from two independent experiments, with error bars representing the standard deviation. The conserved c-maf binding motif (MARE) in the promoter regions of the human and mouse cyclin D2 gene is shown together with the mutated nucleotides of the mutant cyclin D2 construct. The MARE is located between −134 and −122 with respect to the start of translation in the human gene.

D: Surface expression of integrin β7 is shown for cell lines infected with retroviruses expressing either c-maf or a dominant negative form of c-maf. Surface integrin β7 is also shown for H929 cells 72 hr after being transduced with a c-maf siRNA construct.

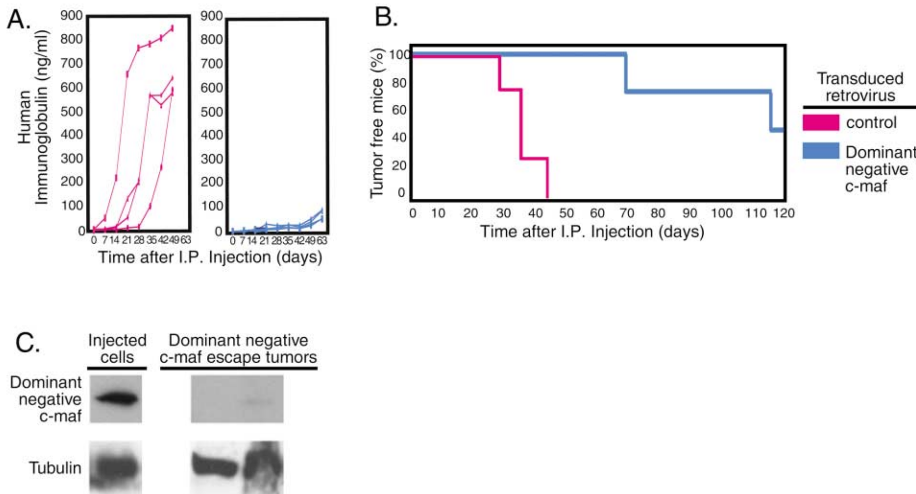

Figure 4. Inhibition of c-maf blocks tumor formation in immunodeficient mice

c-maf-expressing LP-1 cells were transduced with a retrovirus expressing dominant negative Ac-maf or a control retrovirus and injected intraperitoneally into NOD-SCID mice.

A: Human immunoglobulin levels in blood as assessed by ELISA.

B: Occurrence of palpable tumors.

C: Western blot analysis of tumors formed in mice injected with Ac-maf-transduced myeloma cells.

provides another mechanism promoting cell cycle progression in myeloma. The fact that all three D type cyclins are overexpressed in myeloma by disparate mechanisms suggests that forced progression through G1/S phase of the cell cycle is an important and frequent oncogenic event in multiple myeloma.

Whereas most oncogenes render the tumor cell independent of external signals for proliferation and survival (Hanahan and Weinberg, 2000), c-maf defines a class of oncogenes that enhance interactions between stromal cells and tumor cells (Figure 6). This class of oncogenes was predicted to exist (Hanahan

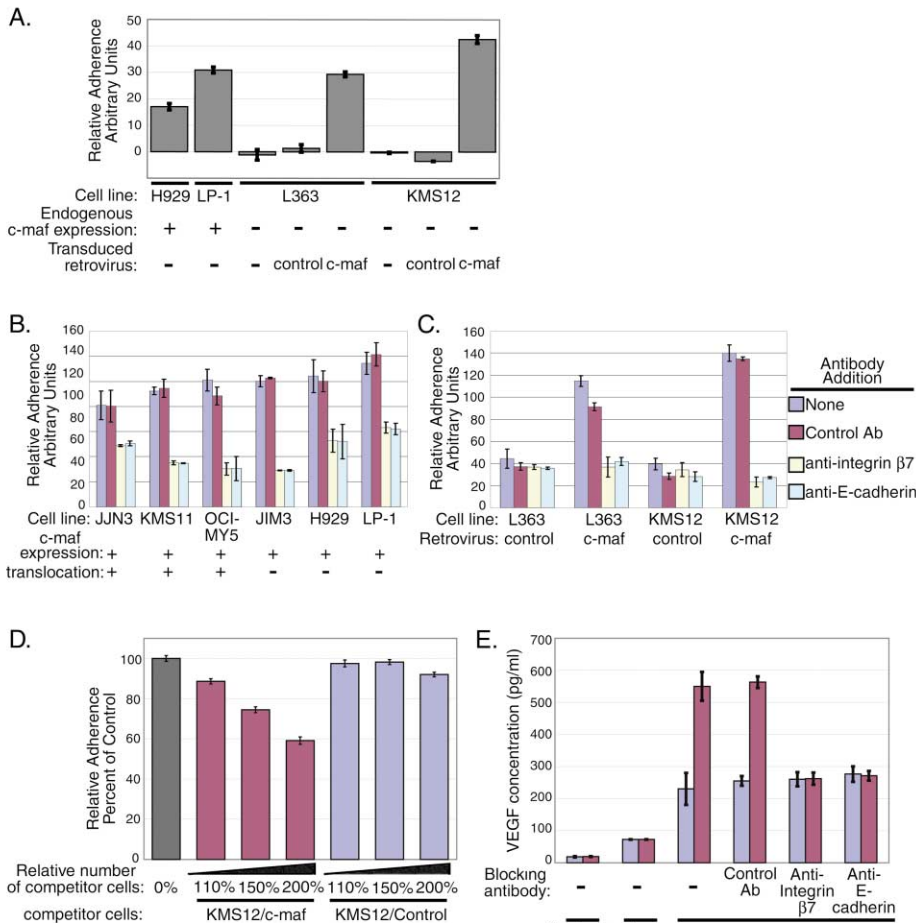

Figure 5. c-maf expression enhances adhesion to E-cadherin and bone marrow stroma

A: Binding of myeloma cell lines expressing c-maf either endogenously or as a result of retroviral transduction to E-cadherin coated plates.

B: Binding of multiple myeloma cell lines expressing endogenous c-maf to bone marrow stroma. The effect of antibodies to integrin β7 and E-cadherin is compared to control IgG1.

C: Binding of myeloma cell lines that lack expression of endogenous c-maf to bone marrow stroma after infection with a retrovirus expressing c-maf or with a control retrovirus. The effect of antibodies to integrin β7 and E-cadherin is compared to control IgG1.

D: Competitive binding assay of myeloma cells to bone marrow stroma. c-maf-negative KMS12 cells were fluorescently labeled and allowed to adhere to stroma in the presence of increasing numbers of unlabeled competitor cells that do or do not express c-maf, as indicated.

E: Secretion of VEGF by KMS12 myeloma cells transduced with control or c-maf-expressing retroviruses, bone marrow stromal cells, or cocultures of myeloma and stromal cells. All assays were done in triplicate and error bars indicate the standard deviation.

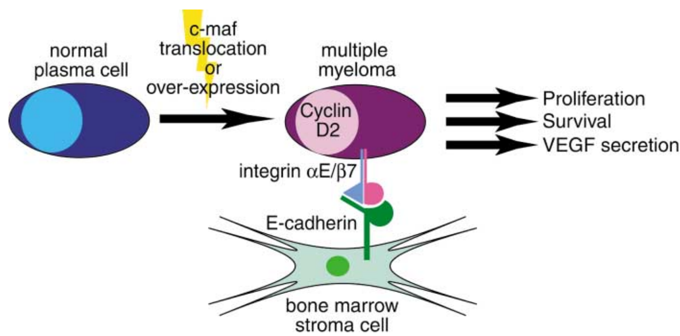

Figure 6. A model by which c-maf overexpression may cause multiple myeloma

See text for details.

and Weinberg, 2000) since, in many cancers, tumor cells receive growth and survival signals from stroma (Liotta and Kohn, 2001; Tlsty, 2001). c-maf translocations occur in MGUS (Fonseca et al., 2002), a premalignant lesion characterized by abnormal accumulation of plasma cells, and thus c-maf influences early events in myeloma pathogenesis. Since bone marrow stromal cells are known to provide survival signals to plasma cells (Kawano et al., 1995; Merville et al., 1996; Minges Wols et al., 2002; Roldan and Brieva, 1991), the enhanced adhesion of c-maf-expressing MGUS cells to stroma may give them a selective advantage over normal plasma cells in the bone marrow microenvironment. Further, enhanced production of VEGF as a result of integrin αEβ7/E-cadherin interactions may function in an autocrine or paracrine fashion to promote myeloma proliferation and survival (Bellamy et al., 1999; Dankbar et al., 2000; Gupta et al., 2001; Podar et al., 2001, 2002). VEGF may also contribute to the enhanced bone marrow angiogenesis that is characteristic of myeloma (Anderson, 2001).

In addition to affecting proliferation and adhesion, c-maf may also alter the function of myeloma cells by upregulating CCR1, a receptor for the chemokine MIP-1α. Indeed, ectopic expression of c-maf in myeloma cells increased their chemotaxis in response to MIP-1α (data not shown). Since myeloma cells from many patients produce MIP-1α (Abe et al., 2002; Uneda et al., 2003), it may be interesting to investigate whether c-maf regulates autocrine stimulation by MIP-1α by upregulating CCR1.

Our observation that c-maf is overexpressed in roughly half of myeloma cases is surprising given the low frequency of c-maf translocations (5%–10%) in myeloma and MGUS (Fonseca et al., 2002; Kuehl and Bergsagel, 2002). It will therefore be interesting to determine the mechanism by which c-maf is overexpressed in cases where no translocation involving the IgH locus was detected. Previous studies have investigated whether the expression of c-maf in myeloma cell lines is biallelic or monoallelic (Chesi et al., 1998). In two cell lines that contained a c-maf translocation, the expression of c-maf was only detected from the translocated allele. However, in the case of LP-1, a cell line that expresses c-maf without a t(16:14) translocation, c-maf expression was detected from both alleles (Chesi et al., 1998). This observation is consistent with a transcriptional activation of c-maf in some multiple myelomas by a currently unknown mechanism(s).

Our results thus identify c-maf overexpression as one of the most frequent oncogenic events in multiple myeloma and establish c-maf as an intriguing novel therapeutic target. Dominant inhibition of c-maf interfered with stromal cell adhesion and proliferation of myeloma cells with c-maf overexpression, whether or not they had a c-maf translocation, and c-maf inhibition blocked tumor formation in immunodeficient mice. Our data predict that an inhibitor of c-maf function would decrease cyclin D2 expression, stromal cell adhesion, and VEGF secretion, thereby inhibiting myeloma proliferation and survival.

Experimental procedures

Gene expression profiling

mRNA from cell lines was isolated using the FastTrack 2.0 kit (Invitrogen). Total RNA from patient samples was isolated using Trizol (GIBCO-BRL). Lymphochip DNA microarrays were prepared, hybridized, and analyzed as previously described (Shaffer et al., 2000). Relative gene expression was assessed by comparing a myeloma cDNA probe (labeled with Cy5 dye) with a reference cDNA probe (labeled with Cy3 dye) prepared from a pool of 9 lymphoid cell lines, as described (Shaffer et al., 2000). DNA microarray gene expression data were used to identify genes coregulated with *c-maf* by comparing expression in c-maf-postive and c-maf-negative cell lines (as determined by quantitative RT-PCR), using a t test to assess statistical significance. Selection criteria for c-maf coregulation were $p \leq 0.001$, variance ≥ 2.0, and <10% missing values. For direct identification of c-maf target genes, Cy5-labeled cDNA probes were prepared from cells transduced with retroviruses expressing c-maf or dominant negative c-maf and compared with Cy3-labeled probes from cells transduced with a control retrovirus. All gene expression data is available at http://lymphochip.nih.gov/hurtetal and as Supplemental Tables S1 and S2 at http://www.cancercell.org/cgi/content/full/5/2/191/DC1.

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

Flow sorting was used to purify plasma cells from bone marrow aspirates of myeloma patients (CD138+, CD38+, CD56+) and from normal donor bone marrow aspirates (CD138+, CD38+, CD19−). Quantitative RT-PCR assays (TaqMan) were performed using a one-step reaction solution using the primers shown below for *c-maf*, *integrin β7*, and *USF2* or using primers provided by Assays-on-Demand (Applied Biosystems). The expression of *USF2* was detected with the forward primer GGAGCGGCCTGCGATTT, the reverse primer CTTTGAAGGTCTCCTGCATGC, and the probe TCCGGGAG TTGCGCCAGACC. *Integrinβ7* was detected with the forward primer GAAT CAACCAGACGGTGACTTTCT, the reverse primer GCCCGGAGCCTCA GGA, and the probe CAAGCCACCCACTGCCTCCCAG. Expression of *c-maf* was detected with the forward primer GCTTCCGAGAAAACGGCTC, the reverse primer TGCGAGTGGGCTCAGTTATG, and the probe CGACAACCC GTCCTCTCCCGAGTTT. The level of *c-maf* and *integrin β7* mRNA was determined by normalization to the level of the control gene *USF2,* which showed little variance in expression across all myeloma cell lines assayed.

Cell culture, retroviral constructs, and transduction

Cell lines were maintained in RPMI-1640 (GIBCO-BRL) or ACL-4 (GIBCO-BRL) with 10% fetal calf serum (HyClone) and penicillin/streptomycin (GIBCO-BRL). Bone marrow stroma was obtained from aspirates of healthy donors and cultured as previously described (Uchiyama et al., 1993). Bicistronic retroviral constructs were generated using the VxyPuro backbone (Shaffer et al., 2000) or the vEGFP-F backbone (Davis et al., 2001) that allow for expression of a cDNA and the puromycin resistance gene or a farnesylated EGFP protein, respectively. Infections were carried out as described (Shaffer et al., 2000), and cells receiving VxyPuro constructs were maintained in puromycin (1 μg/ml). The VxyPuro-cmaf construct contained a human *c-maf* cDNA, corresponding to sequences from −804 to +2005 with respect to the translation start site. The VxyPuro-Ac-maf and vEGFP-Ac-maf constructs were made by fusing a FLAG-tagged acidic extension to the leucine zipper portion of c-maf, +1753 to +2005, as previously described (Olive et al., 1997). To determine the effect of dominant negative c-maf on cell number, myeloma cells were infected with a vEGFP-F retrovirus

expressing dominant negative c-maf or a control vEGFP-F retrovirus, and EGFP positive cells were enumerated by flow cytometry as described (Davis et al., 2001).

Cyclin D2 promoter analysis

Myeloma cell lines were cotransfected with an expression vector (pCMV-Script; Stratagene) containing the c-maf cDNA, a reporter vector in which the human cyclin D2 promoter drives expression of luciferase (Shaffer et al., 2000), and a control β-Gal expressing plasmid, as described (Shaffer et al., 2000). Luciferase activity was measured using a luminometer and levels were normalized to β-Gal expression. Site directed mutagenesis with the mutant cyclin D2 primers, GGGGAGGACCGGgtaGAGTtAGtacGCCCCGA GGC and GCCTCGGGGCgtaCTaACTCtacCCGGTCCTCCCC, was preformed using the QuickChange kit (Stratagene).

siRNA constructs and transduction

The c-maf siRNA duplex construct ACGGCUCGAGCAGCGACAA (Dharmacon) was transduced by electroporation (Amaxa) and live cells were separated by Ficoll centrifugation.

E-cadherin binding assays

Binding was carried out as previously described (Higgins et al., 1998). Briefly, 24-well plates were coated with E-cadherin overnight at 4°C or seeded with bone marrow stromal cells (0.5×10^5). Myeloma cells were incubated with the BCEF-AM dye (Molecular Probes) for 15 min at 37°C followed by washing, incubated at 4°C with blocking antibodies (20 ng/ml), and plated at 3×10^5 cells per well. The plates were incubated a 37°C for 30 min followed by washing and reading in a fluorescent plate reader.

VEGF secretion

Bone marrow stroma, isolated and cultured as previously described (Gupta et al., 2001), was plated at 1×10^4 cells per well in a 96-well plate and allowed to become confluent. KMS12 cells were added at a density of 1×10^3 cells per well. Anti-integrin β7 (Santa Cruz) and anti-E cadherin (Santa Cruz) were added (20 ng/ml) and the plates were incubated at 37°C for 48 hr. Supernatant was collected and assayed for VEGF by ELISA (R&D Systems).

In vivo tumor formation

LP-1 cells were infected with the VxyPuro or the VxyPuro-Ac-maf retroviruses described above. Cells were selected with puromycin (1 μg/ml) for 4 days, washed extensively, and then injected intraperiteonealy into 10-week-old NOD-SCID mice (10^7 cells/mouse). Mice were monitored daily for palpable tumor formation and tail vein blood was assayed weekly for human immunoglobulin lambda production by ELISA (Bethyl Labs). Tumors were removed and protein extracts made for Western blot analysis. c-maf protein was detected by a c-maf antibody (Santa Cruz), and Ac-maf protein, which was engineered to contain a FLAG epitope, was detected by an anti-FLAG antibody (Sigma).

Acknowledgments

We thank M. Aymerich, V. Kapoor, R. Fischer, and L. He for technical assistance.

Received: September 29, 2003
Revised: November 14, 2003
Accepted: December 5, 2003
Published: February 23, 2004

References

Abe, M., Hiura, K., Wilde, J., Moriyama, K., Hashimoto, T., Ozaki, S., Wakatsuki, S., Kosaka, M., Kido, S., Inoue, D., and Matsumoto, T. (2002). Role for macrophage inflammatory protein (MIP)-1α and MIP-1β in the development of osteolytic lesions in multiple myeloma. Blood *100*, 2195–2202.

Anderson, K.C. (2001). Multiple myeloma. Advances in disease biology: therapeutic implications. Semin. Hematol. *38*, 6–10.

Bellamy, W.T., Richter, L., Frutiger, Y., and Grogan, T.M. (1999). Expression of vascular endothelial growth factor and its receptors in hematopoietic malignancies. Cancer Res. *59*, 728–733.

Bergsagel, P.L., Chesi, M., Nardini, E., Brents, L.A., Kirby, S.L., and Kuehl, W.M. (1996). Promiscuous translocations into immunoglobulin heavy chain switch regions in multiple myeloma. Proc. Natl. Acad. Sci. USA *93*, 13931–13936.

Chesi, M., Bergsagel, P.L., Shonukan, O.O., Martelli, M.L., Brents, L.A., Chen, T., Schrock, E., Ried, T., and Kuehl, W.M. (1998). Frequent dysregulation of the c-maf proto-oncogene at 16q23 by translocation to an Ig locus in multiple myeloma. Blood *91*, 4457–4463.

Cook, G., Dumbar, M., and Franklin, I.M. (1997). The role of adhesion molecules in multiple myeloma. Acta Haematol. *97*, 81–89.

Dalton, W.S. (2002). Drug resistance and drug development in multiple myeloma. Semin. Oncol. *29*, 21–25.

Dankbar, B., Padro, T., Leo, R., Feldmann, B., Kropff, M., Mesters, R.M., Serve, H., Berdel, W.E., and Kienast, J. (2000). Vascular endothelial growth factor and interleukin-6 in paracrine tumor- stromal cell interactions in multiple myeloma. Blood *95*, 2630–2636.

Davis, R.E., Brown, K.D., Siebenlist, U., and Staudt, L.M. (2001). Constitutive nuclear factor κB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. J. Exp. Med. *194*, 1861–1874.

Fonseca, R., Bailey, R.J., Ahmann, G.J., Rajkumar, S.V., Hoyer, J.D., Lust, J.A., Kyle, R.A., Gertz, M.A., Greipp, P.R., and Dewald, G.W. (2002). Genomic abnormalities in monoclonal gammopathy of undetermined significance. Blood *100*, 1417–1424.

Gupta, D., Treon, S.P., Shima, Y., Hideshima, T., Podar, K., Tai, Y.T., Lin, B., Lentzsch, S., Davies, F.E., Chauhan, D., et al. (2001). Adherence of multiple myeloma cells to bone marrow stromal cells upregulates vascular endothelial growth factor secretion: therapeutic applications. Leukemia *15*, 1950–1961.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57–70.

Hideshima, T., and Anderson, K.C. (2002). Molecular mechanisms of novel therapeutic approaches for multiple myeloma. Nat. Rev. Cancer *2*, 927–937.

Higgins, J.M., Mandlebrot, D.A., Shaw, S.K., Russell, G.J., Murphy, E.A., Chen, Y.T., Nelson, W.J., Parker, C.M., and Brenner, M.B. (1998). Direct and regulated interaction of integrin αEβ7 with E-cadherin. J. Cell Biol. *140*, 197–210.

Ho, I.C., Hodge, M.R., Rooney, J.W., and Glimcher, L.H. (1996). The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. Cell *85*, 973–983.

Hynes, R. (2002). Integrins: bidirectional, allosteric signaling machines. Cell *110*, 673–687.

Kawano, M.M., Mihara, K., Huang, N., Tsujimoto, T., and Kuramoto, A. (1995). Differentiation of early plasma cells on bone marrow stromal cells requires interleukin-6 for escaping from apoptosis. Blood *85*, 487–494.

Kim, J.I., Ho, I.C., Grusby, M.J., and Glimcher, L.H. (1999a). The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. Immunity *10*, 745–751.

Kim, J.I., Li, T., Ho, I.C., Grusby, M.J., and Glimcher, L.H. (1999b). Requirement for the c-Maf transcription factor in crystallin gene regulation and lens development. Proc. Natl. Acad. Sci. USA *96*, 3781–3785.

Kuehl, W.M., and Bergsagel, P.L. (2002). Multiple myeloma: Evolving genetic events and host interactions. Nat. Rev. Cancer *2*, 175–187.

Kulkarni, M.S., Daggett, J.L., Bender, T.P., Kuehl, W.M., Bergsagel, P.L., and Williams, M.E. (2002). Frequent inactivation of the cyclin-dependent kinase inhibitor p18 by homozygous deletion in multiple myeloma cell lines: ectopic p18 expression inhibits growth and induces apoptosis. Leukemia *16*, 127–134.

Liotta, L.A., and Kohn, E.C. (2001). The microenvironment of the tumour-host interface. Nature *411*, 375–379.

Merville, P., Dechanet, J., Desmouliere, A., Durand, I., de Bouteiller, O., Garrone, P., Banchereau, J., and Liu, Y.J. (1996). Bcl-2+ tonsillar plasma cells are rescued from apoptosis by bone marrow fibroblasts. J. Exp. Med. *183*, 227–236.

Minges Wols, H.A., Underhill, G.H., Kansas, G.S., and Witte, P.L. (2002). The role of bone marrow-derived stromal cells in the maintenance of plasma cell longevity. J. Immunol. *169*, 4213–4221.

Olive, M., Krylov, D., Echlin, D.R., Gardner, K., Taparowsky, E., and Vinson, C. (1997). A dominant negative to activation protein-1 (AP1) that abolishes DNA binding and inhibits oncogenesis. J. Biol. Chem. *272*, 18586–18594.

Podar, K., Tai, Y.T., Davies, F.E., Lentzsch, S., Sattler, M., Hideshima, T., Lin, B.K., Gupta, D., Shima, Y., Chauhan, D., et al. (2001). Vascular endothelial growth factor triggers signaling cascades mediating multiple myeloma cell growth and migration. Blood *98*, 428–435.

Podar, K., Tai, Y.T., Lin, B.K., Narsimhan, R.P., Sattler, M., Kijima, T., Salgia, R., Gupta, D., Chauhan, D., and Anderson, K.C. (2002). Vascular endothelial growth factor-induced migration of multiple myeloma cells is associated with β 1 integrin- and phosphatidylinositol 3-kinase-dependent PKC α activation. J. Biol. Chem. *277*, 7875–7881.

Roldan, E., and Brieva, J.A. (1991). Terminal differentiation of human bone marrow cells capable of spontaneous and high-rate immunoglobulin secretion: Role of bone marrow stromal cells and interleukin 6. Eur. J. Immunol. *21*, 2671–2677.

Shaffer, A.L., Yu, X., He, Y., Boldrick, J., Chan, E.P., and Staudt, L.M. (2000). BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. Immunity *13*, 199–212.

Shain, K.H., Landowski, T.H., and Dalton, W.S. (2000). The tumor microenvironment as a determinant of cancer cell survival: a possible mechanism for de novo drug resistance. Curr. Opin. Oncol. *12*, 557–563.

Tlsty, T.D. (2001). Stromal cells can contribute oncogenic signals. Semin. Cancer Biol. *11*, 97–104.

Tourigny, M.R., Ursini-Siegel, J., Lee, H., Toellner, K.M., Cunningham, A.F., Franklin, D.S., Ely, S., Chen, M., Qin, X.F., Xiong, Y., et al. (2002). CDK inhibitor p18(INK4c) is required for the generation of functional plasma cells. Immunity *17*, 179–189.

Turel, K.R., and Rao, S.G. (1998). Expression of the cell adhesion molecule E-cadherin by the human bone marrow stromal cells and its probable role in CD34(+) stem cell adhesion. Cell Biol. Int. *22*, 641–648.

Uchiyama, H., Barut, B.A., Mohrbacher, A.F., Chauhan, D., and Anderson, K.C. (1993). Adhesion of human myeloma-derived cell lines to bone marrow stromal cells stimulates interleukin-6 secretion. Blood *82*, 3712–3720.

Uneda, S., Hata, H., Matsuno, F., Harada, N., Mitsuya, Y., Kawano, F., and Mitsuya, H. (2003). Macrophage inflammatory protein-1α is produced by human multiple myeloma (MM) cells and its expression correlates with bone lesions in patients with MM. Br. J. Haematol. *120*, 53–55.