Nikhil C. Munshi
Kenneth C. Anderson *Editors*

Advances in Biology and Therapy of Multiple Myeloma

Volume 1: Basic Science

Springer

Advances in Biology and Therapy of Multiple Myeloma

Nikhil C. Munshi • Kenneth C. Anderson
Editors

Advances in Biology and Therapy of Multiple Myeloma

Volume 1: Basic Science

Springer

Editors
Nikhil C. Munshi
Boston VA Healthcare System and
Dana-Farber Cancer Institute
Harvard Medical School
Boston, MA, USA

Kenneth C. Anderson
Department of Medical Oncology
Dana-Farber Cancer Institute
Harvard Medical School
Boston, MA, USA

ISBN 978-1-4614-4665-1 ISBN 978-1-4614-4666-8 (eBook)
DOI 10.1007/978-1-4614-4666-8
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2012951132

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

To my parents Gulab and Chandravadan Munshi who have inspired me and taught me what I know

and

To my wife Medha and sons Vidit and Manit whose love, support, and sacrifice helped me become who I am

Nikhil C. Munshi

To my mentors who sparked my scientific and clinical interest in myeloma

To my fellow researchers and caregivers, with whom I have been privileged to work

To my patients, who are my true heroes and inspire all that I do

And to Cynthia, Emily, David, and Peter for their loving support

Kenneth C. Anderson

Preface

Multiple myeloma has evolved from an incurable disease with no therapeutic options 5 decades ago to a readily treatable disease, based upon increased understanding of its biology and pathogenesis. Nonetheless, myeloma remains a complex disease driven by both genomic and epigenetic alterations. Moreover, interaction of tumor cells with the bone marrow microenvironment confers additional tumor cell growth, and survival advantage, and drug resistance. Advances in our understanding of the pathobiology of the disease have also translated to improved diagnostic and prognostic methods including high-throughput genomics, serum-free light chain, MRI, and PET scanning. Notably, proteasome inhibitors, immunomodulatory agents, as well as other targeted agents, when used singly or in combination, have transformed myeloma therapy and now achieve unprecedented frequency and extent of response. These rapid advances highlight the need for a state-of-the-art resource focused on the biology of myeloma and its clinical application. Our book describes the basic advances in our understanding of the disease biology and delineates molecular mechanisms mediating tumor growth and progression, as well as bone disease and organ dysfunction. Importantly, it provides the preclinical rationale for and clinical efficacy of single and combination targeted therapies directed at the tumor cell in its bone marrow milieu. With an eye toward the future, we update the recent advances using high-density, high-throughput genomic technologies to integrate both DNA and transcriptional changes for improved molecular classification and personalized therapeutic options. Finally, since studies are already reporting prolonged disease-free survival in myeloma, our book highlights the fact that we are now at the threshold of curative outcome in this disease.

Boston, MA, USA

Nikhil C. Munshi, M.D.
Kenneth C. Anderson, M.D.

Contents

Part II Myeloma Microenvironment

Part I
Myeloma Molecular Pathways and Cell Signaling

Chapter 1
Genomic Strategies Determining Progression from MGUS to Multiple Myeloma

Esteban Braggio and Rafael Fonseca

Abstract In the past years we have learned much about the genetics and biology of multiple myeloma (MM) and plasma cell disorders. It is now clear that (nearly) all MM cases are preceded by a benign phase of expansion of monoclonal PCs known as monoclonal gammopathy of undetermined significance (MGUS). It is also known that MGUS is a common condition that increases in prevalence with advancing age. Trying to couple the understanding we have of the genetics of the disease with the specific risk of progression from MGUS to MM could be of importance in determining different risk of progression with associated management strategies. Currently there is limited information regarding the specific factors that drive the progression from MGUS to MM and the risk associated with different genetics classes of the disease. A better understanding of the biologic steps that are needed for progression from a benign stage to a malignant form of the disease is needed, as well as better markers to provide a more dynamic monitoring for incipient disease evolution. In this chapter we discuss some of the background knowledge and basic biology of the disease, and some future strategies for a better surveillance of patients with MGUS.

1.1 Myeloma

Multiple myeloma (MM) is a malignancy that affects nearly 20,000 new cases per year in the USA, and at any given time, there are ~60,000 people living with this disease [1]. Despite many advances in therapeutic options offered, the disease

E. Braggio, Ph.D. • R. Fonseca, M.D. (✉)
Mayo Clinic in Arizona,13400 East Shea Boulevard, Collaborative Research Building, 1-105, Scottsdale, AZ 85259-5494, USA
e-mail: fonseca.rafael@mayo.edu

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_1,

remains lethal for the majority of patients. Recent studies have shown at least doubling, if not tripling, of the expected median survival time for patients with MM with the advent of novel therapeutics such as bortezomib and lenalidomide, alone or in combination with other agents such as thalidomide, alkylators, and corticosteroids [2–12]. Better approaches for the early detection of MM as well as for the generation of strategies to prevent the progression from MGUS to MM are needed as they would reduce the morbidity associated with disease progression and would allow for a better quality of life with early treatment of those at imminent risk for disease progression.

MM is characterized by the expansion of monoclonal plasma cells (PCs), mostly restricted to the bone marrow (BM) microenvironment for their survival. In most patients, the presence of circulating monoclonal PCs is at very low numbers, thus not detectable through the standard clinical testing (e.g., hemogram). Plasma cells are detected at high numbers in the blood only in cases of very aggressive MM such as in plasma cell leukemia (PCL).

Monoclonal PCs are characterized by the production of monoclonal immunoglobulins [13]. In all cases, PCs produce at least a light chain (kappa more commonly than lambda) and frequently a heavy chain (mainly IgG and in decreasing proportion IgA, IgD, and IgE). The heavy chain is secreted from the cells into the BM interstitial fluid and ultimately reaches the circulation and allows for monitoring for the disease as well as responsiveness to treatment. The test that has been classically used for the detection of monoclonal immunoglobulins is the serum protein electrophoresis and is widely available as a routine clinical test, often leading also to the diagnosis of MGUS. Multiple additional assays exist for the determination and measurement of these monoclonal proteins [immunofixation, quantitative immunoglobulins, serum free light chains (FLCs), and urine tests] that will not be further discussed in this chapter [14].

As a consequence of the growth of monoclonal PCs in the BM, once the disease becomes malignant, patients will have a reduction in normal hematopoiesis resulting in anemia and other cytopenias [15–18]. Thrombocytopenia is uncommon except in cases with very advanced plasmacytosis and extensive replacement of the BM by clonal PCs. As discussed elsewhere in this book, the clonal PCs produce substances that affect the normal bone metabolism resulting in bone loss, manifested as osteoporosis, lytic bone lesions, and the associated consequences including bone fractures [19]. Additionally, patients can also have other organs damaged, particularly renal insufficiency, as a consequence of the clonal growth and the production of monoclonal immunoglobulin [15–17, 19, 20]. The circulating light chains are small enough that they can be filtered into the tubular structures of the kidney, causing cast nephropathy and renal failure in a subset of patients with MM. The treatment of the disease can prevent further deterioration of bone lesions, but rarely do these lesions completely heal once they have been established. Patients can have improvements in bone stability as a consequence of sclerosis of the rims of those lesions as well as healing associated with some of those fractures; however, loss of natural function is common after a fracture secondary to MM. One of the complications that can be irreversible in patients with MM is the development of renal failure. While the failure

can be reversible if treated soon after it is established, patients who present with more protracted pictures will have difficulty in reversing the renal function and frequently will ultimately require hemodialysis. Conversely, the development of anemia or hypercalcemia can usually be reversed after medical interventions such as transfusions, intravenous fluids, or the use of steroids.

It is the presence of these bone lesions and renal failure that are dreaded complications of MGUS progressing to MM and that would be desirable to prevent at the preceding stage. As will be discussed below, however, it is currently difficult to know which patients are at risk for progression from MGUS to MM. A future where patients can be readily identified when they are at imminent risk of progression would be ideal, given that early treatment potentially could delay the development of MM and certainly would be useful in preventing organ damage, such as bone lesions and renal failure. Better management strategies are needed for the monitoring and surveillance of these patients.

MGUS is characterized by the clonal expansion of PCs inside the BM but without harm to the patient. The difference with MM is that this clonal expansion is discreet enough such that the PCs did not cause negative effects on the surrounding hematopoietic cells and patients therefore have no anemia. Likewise, patients have no evidence of lytic bone deletion or bone destruction and usually the protein production is low enough that the patients will not have renal failure [21]. MGUS is present in ~3% of the population over the age of 50, and the prevalence increases with advancing age [21, 22]. For practical purposes, it is important to differentiate those MGUS of the IgM type versus those that are not IgM. The IgM-type MGUS is derived from lymphoplasmacytic cells instead of from PCs and put the person at risk for the development of lymphoid disorders such as Waldenström's macroglobulinemia but not MM [21]. Relevant to this chapter are those gammopathies that are IgA, IgG, or light chain only. The group at Mayo Clinic in Rochester has recently described the presence of an MGUS variant characterized exclusively by the presence of circulating light chains with no heavy chain attached [23]. Biologically, these cases are likely to be variants of other PC clonal expansion processes and will also likely include the similar genetic abnormalities in those cells.

Clinically, the management of patients with MGUS includes routine surveillance to detect early signs of disease progression. However, the criteria that determine progression remain largely subjective and are often fraught with clinical nuances that make it difficult to determine which patients are in need of therapy versus those that are not. Extreme clinical situations are easily identified such as a patient with no evidence of complications, whatsoever, versus a patient who has clear need for treatment. However, frequently the clinician may encounter patients who have intermediate stages of a mild anemia or mild renal insufficiency where a subjective determination has to be made regarding the need for therapy. In addition, it is conceivable that some patients that present with a light chain that is particularly damaging to the kidney will result in a phenotype of renal failure despite a clonal process that is rather quiescent. It is then that the clinical classification of MM complications does not necessarily fully correspond to the stages of clonal evolution present inside the BM.

Patients are usually reassured of the benign nature of the disease when they receive a diagnosis of MGUS. These patients will be counseled to have at least a yearly determination of their monoclonal protein as well as measuring hemoglobin, calcium, and creatinine levels. However, the optimal strategy for determining the patient at imminent risk for progression or detection of early progression is not well defined. Recent studies have identified that patients with high concentration of monoclonal protein, serum FLCs, and ultimately indirect markers of increased tumor bulk are at greater risk for disease progression [24, 25]. Results showed that the risk of progression in patients with abnormal FLC κ/λ ratios was significantly higher than that in patients with normal ratios. Furthermore, the risk of progression increased as κ/λ ratios became more extreme. It is our hypothesis that this greater risk of progression results from a higher probability of acquiring a secondary genetic abnormality (stochastic) since there are a greater number of cells at risk for disease progression.

Like MM, MGUS is more common in individuals of African origin. Population-based studies have shown that MM appears to be at least two times more common in individuals of African origin than in individuals of Caucasian descent [26, 27]. A recent study conducted in Ghana showed that patients there have twice the prevalence of MGUS as do patients in the USA, indicating that the higher prevalence of MM in Africans is not due to a higher rate of disease progression, but rather due to a higher propensity for developing MGUS [27]. Likewise, another study looking at serially collected sera from two patient populations has conclusively shown that all MM patients are preceded by an MGUS stage [28, 29]. It is then that we understand that MGUS is of paramount importance to understanding MM. Furthermore, the majority of genetic abnormalities observed in MM are also observed in MGUS, and the specific prevalence and implications will be discussed below. By performing comparative longitudinal genetic analysis between the clonal cells of MGUS and MM, we would expect to be able to elucidate those genetic factors that result in more rapid cell growth or ineffective apoptosis ultimately leading to clonal expansion and a malignant phenotype.

1.2 The Genetics of Plasma Cell Disorders

Over the past 15 years, much progress has been made in understanding the genetic basis associated with the clonal expansion of PCs. At the very top level, two major genetic subtypes of MM can be found: the so-called hyperdiploid MM (H-MM) and the non-hyperdiploid MM (NH-MM) [30–32]. The H-MM variant is characterized by the presence of multiple trisomies, particularly affecting the odd-numbered chromosomes 3, 5, 7, 9, 11, 15, 19, and 21 [31, 33–36]. The recurrent pattern observed suggests that the presence of extra copies of these specific chromosomes somehow results in a favorable environment for the clone, allowing further expansion and proliferation. Patients with H-MM can have a number of other genetic abnormalities such as gains of 1q and deletions of 1p, 13, 17p, as well as *IgH*

translocations. However, the overall majority of patients with H-MM will not have *IgH* translocations, which are the hallmark of the NH-MM as will be discussed below. Indeed, if the H-MM have *IgH* translocations, they are likely to belong to either the t(4;14) group or patients with the less common forms of the *IgH* translocations. Several studies have shown that the presence of H-MM is an early event in the clonal expansion of PCs and can be readily detectable in cases of MGUS [37–39]. Furthermore, the presence of H-MM has been associated with a more favorable outcome among MM patients [40–42]. Patients with H-MM have a propensity to have a higher extent of bone disease and tend to be older than those patients without H-MM. It is currently unknown whether there is a negative or a positive effect on the likelihood of progression from MGUS to MM in patients who have H-MM.

The other main genetic subgroup is the NH-MM. This genetic subtype of the disease, which comprises approximately half of patients, is mainly characterized by the presence of *IgH* translocations. These translocations are thought to be seminal events in the pathogenesis of the disease and involve an array of recurring chromosomal partners. The most common chromosomal translocations include the t(4;14) and the t(11;14) present in about 15% of patients each, and the t(14;16) present in ~5% of patients. In lower frequency are found the t(6;14) and the t(14;20) present in 3–5% of patients each. There are a number of other translocations, which are less common [43–45]. These translocations are present since the early stages of the disease and when present are detectable in a large majority of clonal PCs [42, 46]. In fact, those clonal cells without the translocation likely are not displaying it due to technical issues. Nevertheless, a recent paper suggested that the *IgH* translocations could be present in a lower percentage of clonal cells, and if indeed confirmed, this would have profound implications regarding the importance of chromosomal translocations in the pathogenesis of the disease [47]. Nevertheless, the vast majority of clinical studies published so far shows that the majority of clonal cells harbor these chromosomal translocations [42, 46, 48].

1.2.1 Translocation t(11;14)

The t(11;14) results in the upregulation of the *CCND1* gene and, in general, results in a more favorable outcome [42, 46, 49–51]. Nevertheless, there are certain subtypes of MM with the t(11;14) that display a more aggressive behavior. Interestingly, the cells that harbor this translocation frequently display lymphoplasmacytic morphology with scant cytoplasm [52, 53]. It is also notable that other malignancies affecting late-B cell and PCs are also enriched for this translocation. In particular, a large fraction of patients with primary PCL can harbor the t(11;14) as well as patients with IgM-variant MM [54]. It is also remarkable that nearly half of patients with light chain-associated amyloidosis, a disease state that can result from a minimal plasmacytosis, will harbor this translocation [55]. The translocation is found in patients with remarkable genomic stability showing few other changes on top of the t(11;14).

1.2.2 Translocation t(4;14)

The t(4;14) results in the upregulation of the *FGFR3* and *MMSET* genes. This translocation has been associated with a more aggressive clinical behavior and has been associated with a shorter survival [42, 46, 50, 56, 57]. On the other hand, patients with this translocation have a lower prevalence of lytic bone lesions. These patients had a shorter progression-free survival when treated with standard chemotherapy followed by a single autologous stem cell transplant [46, 50, 58, 59]. It appears that this group of patients benefits from the addition of bortezomib as part of the first-line therapy of the disease, although when this translocation is used as a prognostic factor for groups treated with bortezomib, it still retains its prognostic significance [5, 60]. Early attempts at blocking the expression of *FGFR3* associated with this translocation have been unsuccessful, although elegant preclinical work has shown that targeting *FGFR3* may be of benefit in the management of MM patients with this specific abnormality [61, 62].

1.2.3 Translocation t(14;16) and Other MAF Translocations

The t(14;16) results in the upregulation of the *MAF* proto-oncogene [63]. This translocation is quite similar to the t(14;20), which results in the upregulation of *MAFB*. In several clinical series, the t(14;16) has been associated with a more aggressive behavior and shorter survival [42, 64]. A similar inferior outcome was also associated with t(14;20), affecting *MAFB* [65]. A recent study questioned the usefulness of t(14;16) as a prognostic marker, but at least four other studies have shown that patients with this abnormality have a shorter survival [66]. Even in the study that questioned its prognostic value, patients with t(14;16) had a higher number of circulating PCs, something that has been described as an adverse prognostic factor in MM. We have recently reported that patients with this translocation have absent expression of NCAM [67], something that could explain a higher propensity of PCs with the t(14;16) to be circulating in extramedullary sites and locations such as the peripheral blood [68]. No specific therapeutic strategies have been developed so far for the management of patients with t(14;16).

1.2.4 Deletion of Chromosome 13

Most cases with abnormalities in chromosome 13 are characterized by monosomy 13 (~85%), whereas the remaining 15% of cases have interstitial deletions [69, 70]. Deletion of chromosome 13 was initially associated with shorter survival in patients with MM [71–74]. It is now clear that this effect was the consequence of deletion 13 being an indirect marker for NH-MM. In particular, chromosome 13 is enriched in

patients with the t(4;14) and the t(14;16) and therefore preselected patients with aggressive variants of the chromosomal translocations [32, 46, 56]. Thus, at least 80% of patients with t(4;14) will also harbor deletion 13. It is no longer considered a high-risk genetic marker, although patients with this abnormality in general tend to have a shorter survival because of the association with the aforementioned translocations. Deletion 13 nevertheless appears to be important in the pathogenesis of the disease, as it is clonally selected and present in the majority of the clonal PCs [69]. The exact genes involved in the pathogenesis of MM associated with chromosome 13 have not been fully elucidated, although most genetic mapping studies continue to point at *RB1* as a suspect gene associated with deletion 13.

1.2.5 Deletion of 17p13

Deletions of the short arm of chromosome 17 are also thought to be important in the pathogenesis and progression of MM. Indeed, deletion of 17p13 remains the single most important genetic prognostic factor in MM [42, 46, 64, 75]. As in the case of deletion 13, the exact gene(s) have not been fully elucidated, although a body of data suggested *TP53* being the critical gene. Deletion of 17p13 is present in ~10% of patients but increases in prevalence with advancing stages of the disease [42, 76]. We have recently found that this abnormality is present in 20% of MM patients at the time of their first relapse and 30% of the patients at second relapse and later. Furthermore, we have shown that patients with PCL [76] as well as human myeloma cell lines (HMCLs) have a very high prevalence of chromosome 17 deletions and *TP53* mutations [77]. In contrast, deletion 17p13 appears to be quite rare in patients with MGUS and smoldering MM (SMM). In our series, we have found only one MGUS patient with deletion 17, and the prevalence in SMM disease has been estimated at 3%. While only longitudinal and prospective studies can fully address the question as to whether deletion 17 could be associated with the progression of the disease, the cross-sectional evaluation leaves no doubt that the acquisition of deletion 17 is a progression event, and therefore a patient in early stages of the disease with this abnormality should be considered at potential risk of earlier disease progression.

1.2.6 Chromosome 1

Copy number gain of 1q21 is among the most commonly reported genetic abnormalities seen in MM cases [31, 40, 78, 79]. A putative target of this amplification is *CKS1B*, which promotes the degradation of p27, an inhibitor of cell cycle progression. Previous studies suggest that gain of 1q21 is associated with both disease progression and poor prognosis [80]. It was shown that the prevalence of 1q abnormalities goes from 0% in MGUS to 43% in newly diagnosed MM and 72% of

relapsed MM [80]. Patients with 1q21 copy number gain have a higher prevalence of deletion 13 and t(4;14). A published high-risk genetic signature for MM is highly enriched for genes located on chromosome 1, including *CKS1B* between them. Even though the gain of 1q21 has been associated with poor prognosis in MM [79, 80], its analysis has not been implemented into the routine clinical practice yet and its effect is still under investigation.

1.2.7 RAS Mutations

Mutations affecting *RAS* genes are present in ~40% of MM and HMCLs [81–83]. Remarkably, they have been identified as involving both *KRAS* and *NRAS* at codons 12, 13, and 61, but not *HRAS*. Activating mutations on *RAS* genes appear to be mutually exclusive with mutations on *FGFR3*, suggesting that activating mutations affecting both genes have a similar effect [81].

Only those mutations affecting *KRAS* have been associated with a shorter survival. However, it is possible that *NRAS* mutations are important in the progression from a quiescent benign state of PCs to a more aggressive phenotype associated with MM. Some studies have failed to show the presence of *RAS* mutations in MGUS and therefore could indicate a role for this mutation accelerating clonal growth in the progression of MGUS to MM.

1.2.8 Gene Expression Profiling

Although the use of gene expression profiling (GEP) data has been successfully implemented as a risk-stratification tool in MM [64, 84, 85], the same did not happen in the identification of progression markers from MGUS to MM. A critical point to be highlighted is that a successful GEP experiment depends on the high purity of the tumor population. In MM, it is assumed that the majority of CD138+ cells (marker of PCs) are clonal, thus being accepted as the routine technique for tumor purification. That is not necessarily the case in all MGUS, where in some cases the normal PCs still represent a significant proportion of PCs found in BM. Recently, six-color sorting strategy has been implemented with the goal of identifying the clonal cells and to help set the gates that will separate the clonal plasma cells from normal polyclonal counterparts [86, 87]. A comparative GEP study between the proper clonal population of MGUS and MM might be extremely powerful in the identification of disease progression markers.

1.2.9 High-Throughput Mutation Analysis

Recent whole genome sequencing analyses have led to the confirmation of mutations in genes previously described in MM such as *RAS*, *TP53*, and regulators of the NF-kB signaling pathway as well as to the identification of mutations in previously unaffected genes and pathways in MM [88]. The frequency of some salient mutations is as follows: *CCND1* 2%, *BRAF* 4%, *DIS3* (RPP44) 11%, *FAM46C* 13%, *XBP1* 4%, *LRRK2* 6%, *IRF* 6%, and *PRDM1* in 6%. Other notable findings were the identification of pathway-specific mutations such as genes associated with protein translation 42%, histone-modifying enzymes (*HOXA9* and others) and fibrin clot formation genes in 16% of cases.

The lack of conclusive longitudinal studies that can address the role of genetic factors as progression events from MGUS to MM is due to the fact that BMs are not frequently done in patients with MGUS and that a very large number of patients need to be studied, given the rarity of progression events. The rate of progression from MGUS to MM has been estimated at ~1% per year, and therefore to achieve enough statistical power, factors that have a high relative risk of progression and a large cohort of patients need to be analyzed.

1.2.10 Epigenetic Factors

Most of the analyses focused on the study of epigenetic abnormalities in MM have been performed on single locus [89–93]. Inactivation of *CDKN2A* (p16) and other genes by promoter hypermethylation has been studied also as potential markers for progression from MGUS to MM [89]. The methylation level appears to be progressive with advancing stages of the disease and could be considered one of the hallmarks for disease progression and poor outcome [94, 95]. Methylation of *CDKN2A* appears to be more common in MM than in MGUS, but again the studies are not conclusive to categorically state that methylation of specific genes is associated with disease progression. Other studies have looked at other sets of genes, which remain hypothesis generating, but none so far have been able to conclusively show the risk of progression associated with their presence [89–93].

A recent study focused on the WNT pathway showed the utility of examining sets of genes rather than single genes in MM pathogenesis [96]. Only a small percentage of patients showed hypermethylation of the genes encoding these ligands; however, when various genes on the pathway were analyzed, 42% of patients showed methylation and silencing of at least one of the seven genes examined in myeloma.

By using high-throughput approaches, we and others have shown differences in the methylation profile between MGUS and MM. This transition was mainly characterized by the overall hypomethylation of the genome and the gene-specific hypermethylation at the transition from MGUS to MM stage [97, 98] although the exact mechanisms leading to this hypomethylation state are currently unknown. In addition, DNA methylation profile has shown significant differences between

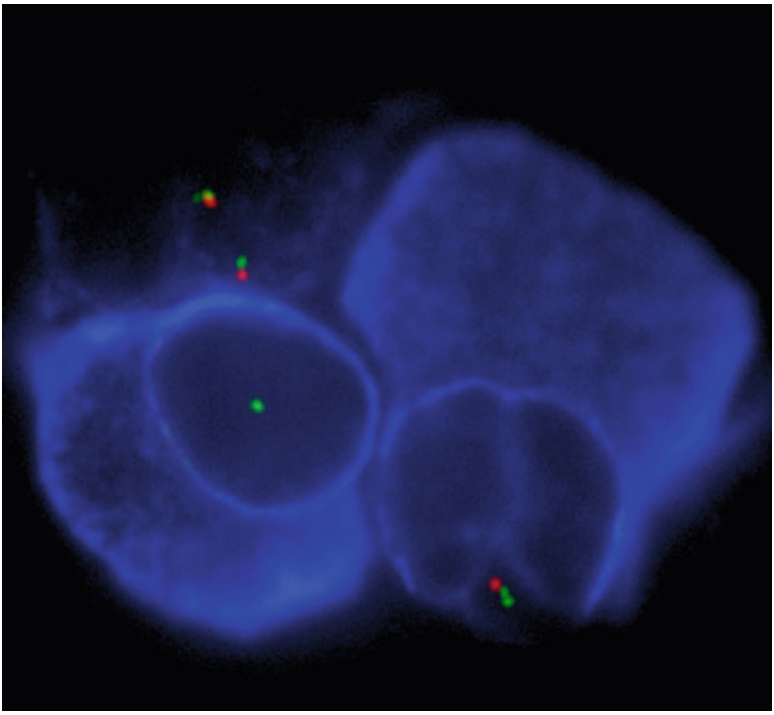


Fig. 1.1 Example of clonal diversity in MM identified by single-cell interphase FISH analysis. At the *top*, a non-PC (negative for cIg-staining) with two *red* (R) and two *green* (G) FISH signals is shown. At the *bottom*, two PCs with different genetic arrangements (0R1G and 1R1G, respectively) are shown

cytogenetic subgroups [97]. More specifically, methylation subgroups were defined by translocations and hyperdiploidy, with t(4;14) myeloma having the greatest impact on DNA methylation [97].

1.2.11 Subclonal Heterogeneity

It is now clear that while all MM cells, in any given patient, share common ancestry, they also diverge and drift genetically with disease evolution [99–101]. The subclonal heterogeneity, once identified, can be analyzed using FISH or deep sequencing in single-cell analysis (Fig. 1.1) [102, 103]. The presence of resistant subclones at the time of diagnosis will result in only temporary disease control and ultimate proliferation of cells with a growth and survival advantage. In addition, this subclonal heterogeneity will change on clonal selection pressures associated with the various therapeutic approaches. The ultimate cure of the disease can be achieved only by eliminating all potential subclones containing "driver" mutations at the time of initial therapy.

1.3 Evaluating Disease Progression

Currently, few markers are available to identify disease progression from MGUS to MM. In that regard, longitudinal studies of genetic abnormalities between disease stages have been useful in generating the basic, although fragmented, genetic landscape of disease evolution. Thus, several genetic abnormalities are shared between MGUS and MM, indicating their status as early events on the disease. Conversely, another subset of genetic changes is absent in MGUS and found only in more advanced phases of the disease, thus serving as markers of disease progression. With our current understanding of MM, we propose the following model for disease initiation and progression.

The major division on two genetic groups found in MM (H-MM and NH-MM) is already detected in MGUS, thus indicating that both groups of genetic abnormalities are initiating events in pathogenesis [38, 39, 104]. Like MM, nearly half of the MGUS patients have *IgH* translocations. In addition, several studies have shown that the prevalence of the t(11;14) and t(14;16) is comparable between MGUS and MM [105]. That is in agreement with the hypothesis indicating that the primary *IgH* translocations occur during the IgH switch recombination in the germinal center during the maturation of the late-B cell [106]. Although the t(4;14) is also observed in MGUS, it seems to be less common and present more frequently in patients with SMM and MM [39, 104]. The deletion 13 is already present in MGUS and its prevalence has been varied between 25 and 50% of MGUS cases across studies [39, 48, 105, 107, 108] and has been recently suggested that the prevalence of deletion 13 was related to the presence of *IgH* translocations [105].

Similar to *IgH* translocations, the prevalence of aneuploidy is independent of disease stage. Thus, the remaining 40–50% of MGUS cases are characterized by an aneuploid karyotype [37, 38, 105, 109, 110].

Conversely, a subset of genetic events is clearly associated with disease progression. One of the best characterized is the deletion of 17p13. Deletion of 17p13 and mutation of *TP53* are present only in 0–2% of MGUS [111–113], but its prevalence increases in later stages of the disease going to 10% in newly diagnosis MM, 20% at the time of the first relapse, 30% at second or third relapse, and 50% in PCL [76]. Furthermore, deletions and mutation of *TP53* are observed in a vast majority of HMCLs [77].

Another genetic markers associated with disease progression are chromosome 1p loss and 1q gain. Chromosome 1 abnormalities are highly prevalent in newly diagnosed and relapsed MM but absent or found in very low prevalence in initial stages of the disease [80]. Recently, a serial analysis has shown that the 1p loss was acquired during the progression from SMM to MM [114].

Upregulation of *MYC* has been also frequently identified as a genetic event found in progressive disease, mainly involved in secondary IgH translocations [106, 114–116]. However, in a recently generated mice model (Vk*MYC), the AID-dependent *MYC* activation in germinal center B cells was associated with the progress to an indolent

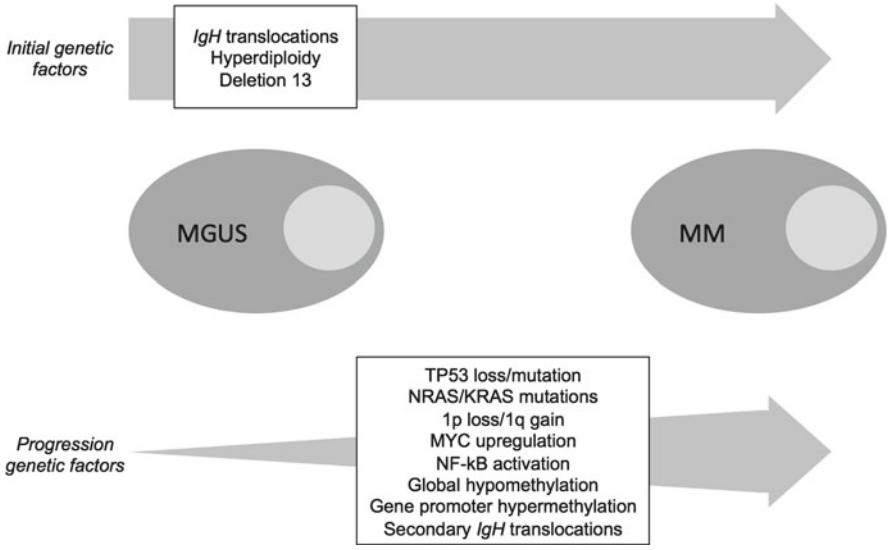


Fig. 1.2 Schematic representation of the temporal acquisition of genetic abnormalities in the progression from MGUS to MM. The initial genetic factors occur in premalignant B cells previous to MGUS. The progression genetic factors are de novo events or show a marked prevalence increase in the transition from MGUS to MM

MM stage sharing the biological and clinical features highly characteristic of the human disease [117]. These data suggested that *MYC* dysregulation could have a causal role in the progression of MGUS to MM.

Activating mutations of *KRAS* and *NRAS* have been also associated with progression from MGUS to MM. Of interest, RAS mutations are enriched among patients with t(11;14), thus being more likely important factors for disease progression for this subtype [118, 119].

There are other recurrent genetic and epigenetic events without a clear association with the progression of the disease. We and others have recently found mutations affecting multiple regulators of the NF-kB signaling pathways, ultimately leading to the constitutive activation of those pathways [120, 121]. It is not very clear their relationship with disease progression although are likely secondary genetic events.

The ideal analysis to better elucidate the real power of these markers to predict progression from MGUS to MM would be a longitudinal analysis including same patient samples corresponding to MGUS and MM stages. In the meantime, the aforementioned genetic events are utilized, in less or more extent, to understand the steps involved in disease progression from MGUS to MM as well as used as disease prognostic markers. A schematic representation of the current state of art is shown in Fig. 1.2.

1.3.1 In Vitro and in Vivo Models of Disease Progression

Recapitulating progression from MGUS to MM is difficult in the laboratory given all the nuances of disease progression in the clinic. The differential growth rate for clonal PCs in the BM could be small enough over a period of many years such that seemingly trivial differences could account for different risk of progression. The patterns of progression from MGUS to MM are variable, but some patients have a steady but continuous to rise in the concentration of their monoclonal protein and PCs, indicating only a very slight differential in the growth rate of the clone that ultimately results in organ damage. In contrast, there are other patients who appear to acquire a secondary genetic event that would lead to a rapid increase in the number of cells resulting in disease phenotype. Trying to recapitulate this in the laboratory remains artificial at best. Cell culture systems that would result in dramatic growth changes probably will have no applicability in the clinic given the very long latency of the disease and the progression from MGUS to MM that occurs sometimes over decades. Likewise, while animal models can clearly identify the additive effect of specific genetic changes in the process of clonal evolution, this still remains limited in its relevance and applicability to the human disease. It seems that, for the time being, only cross-sectional comparison of patients with MGUS versus those with MM will have the power to yield clues regarding the acquisition of secondary genetic hits for disease progression.

1.3.2 Progression of the Disease, Intrinsic Versus Extrinsic

It could be argued that progression from MGUS to MM could be extrinsic and not necessarily derived from the acquisition of secondary genetic hits for the MM clone. For instance, changes in the microenvironment or changes in the immune surveillance process of the host could allow for a more rapid growth of clonal PCs leading to the phenotype of MM. In fact one such study suggests that a decrease in immune surveillance could allow for a more rapid growth of PCs resulting in a phenotype of MM. Our hypothesis remains that the progression process is intrinsic and not necessarily related to external factors of the microenvironment. While there is no doubt that the microenvironment remains an important part in the survival and maintenance of clonal PCs, it is still not clear whether it participates actively in the process of disease evolution. Likewise, it is unclear whether features such as angiogenesis or other growth factors play any role if any in the evolution from the benign stages of the disease.

One of the best clinical models to prove this point of intrinsic progression is the biclonal MGUS. Patients with biclonal gammopathies will frequently have discreet subpopulations of PCs in the BM, each one of which can produce different monoclonal proteins. Genetic studies done in biclonal gammopathy cell populations have revealed that one can identify populations of cells that shared a common ancestor,

Table 1.1 Main clinical and genetic differences observed between MGUS and MM

	MGUS	Newly diagnosed MM
Clinical presentation		
PCs in BM	<10%	>10%
Monoclonal protein in serum	<3 g/dL	>3 g/dL
Organ damage	No	Yes
Genetic factors		
NRAS/KRAS mutations	<5%	~40%
TP53 loss/mutations	<2%	~10%
1p loss/1q gain	<5%	~40%
MYC upregulation	No	Yes
Global hypomethylation	No	Yes
Gene promoter hypermethylation	+	++

as would be determined by the presence of shared genetic abnormalities, yet at some point one of those cells started producing a second type of monoclonal immunoglobulin presumptively as a consequence of IgH rearrangement. Clinically, when these patients are followed and they progress to MM, it can readily be identified that the progression occurs with only one of the monoclonal proteins rising and the other one decreasing in concentration. One would presume, if progression was extrinsic, that you would see growth of both types of cells assuming there is no differential effect of the microenvironment on the soft populations of cells that experience progression. Conversely, if progression is intrinsic, one would expect that stochastically one of the cells will acquire a secondary genetic hit, will exhibit more rapid growth or less apoptosis, and ultimately will become the dominant clone. While this is difficult to prove experimentally, we are still led to believe that progression from MGUS to MM is primarily an intrinsic process driven by the acquisition of secondary genetic hits although this has not been fully elucidated. The more salient differences comparing MGUS to MM are depicted in Table 1.1**.**

Another theory is that the BM has a niche that can contain only a certain number of PCs beyond which it is not capable to maintain. Factors changing in the microenvironment of potentially surveillance cells could allow for a more rapid growth of PCs and expansion beyond their designated physiologic niche. Nevertheless, this theory would not seem to be supported by the monoclonal progression of biclonal MGUS.

1.3.3 Clinical Monitoring for Progression

Despite the reassurance of clinicians to patients who have MGUS, the process for monitoring and surveillance remains imperfect. The optimal interval for testing is

unknown, and retrospective analysis of large cohorts of patients with MGUS suggests that the process is fraught with problems and is unable to detect early signs of progression in the majority of patients. Given the current status of our knowledge of these genetic factors, it is unlikely that genetics alone will be able to predict those patients at more imminent risk of progression. Static formulas that only take into consideration markers of tumor burden and features of disease biology (e.g. genetic events) only provide a limited approximation of the total risk of progression and their clinical usefulness will likely remain limited. It seems more appealing to develop systems that are able to detect in a dynamic fashion early signs of clonal evolution. This could be a system that incorporates measurements that would take into account changes in the concentration of the monoclonal protein, changes in the function of organs potentially affected with MM, or more sensitive markers of bone metabolism. Perhaps it will be the integration of many of these markers at more frequent intervals that would allow for the earlier treatments of patients at imminent risk for progression. It seems that it would be a clinically worthwhile intervention to have systems such that a patient at imminent risk of progression is started on treatment earlier, spared of the quality of life detrimental complications such as bone disease and renal failure.

1.3.4 Public Health Implications

All of these studies have to take into consideration that MGUS is a very common condition and frequently this will not be initially cared for a hematologist. MGUS has become an ancillary diagnosis in elderly patients who have many medical problems and therefore automated, and more reliable systems for surveillance and monitoring are needed. Education of primary care providers will be essential so that patients who show incipient signs of progression from MGUS to MM will become carefully followed up and will receive early intervention. Because of the complexity of integrating biomarkers, genetic markers, and do so in a dynamic fashion, it is likely that coordination of monitoring will best be done through referral centers or through automated systems that can provide an index that will identify a patient with a high risk of progression. A brighter future would be one where a patient who has evidence of clonal expression of PCs could be more accurately predicted in risk of progression and could receive the proper interventions at the time of incipient progression.

1.4 Conclusion

Optimal management of patients with MGUS and at risk for MM progression will likely be a combination of baseline risk factors plus a system that allows dynamic monitoring for disease progression. These baseline factors can be genetics and clinical markers in nature. It seems unlikely that one set of factors alone will have ultimate

power in completely predicting clinical variability. Therefore, coupling a baseline (cross-sectional) analysis of risk with dynamic monitoring will be of key importance in avoiding patients who at the time of diagnosis suffer from irreversible MM complications.

In addition to its predictive abilities, the identification of MM molecular pathways of progression also holds the promise of genetic or pathway targeting strategies that will result in better treatments (i.e., the Achilles heel of the disease). Myeloma has such genetic complexity that single agent, small molecule-based, strategies would seem unlikely to result in durable clinical remissions, such as is the case in chronic myelogenous leukemia (CML), where long duration of disease control is now possible with the use of single agents such as imatinib. However, the situation is quite different in accelerated phase CML, where patients fared better with an allogeneic stem cell transplant. In some cases, MM will be treated as chronic CML, but perhaps most commonly as accelerated phase CML. If that is the case, genetic-targeted therapy in combination with the currently available novel agents is likely to be the most promising treatment strategy.

References

1. Jemal A, Si egel R, Xu J, Ward E (2010) Cancer statistics. CA Cancer J Clin 60:277–300
2. Rajkumar SV (2010) Optimising bortezomib in newly diagnosed multiple myeloma. Lancet Oncol 11:909–910
3. Rajkumar SV, Jacobus S, Callander NS et al (2010) Lenalidomide plus high-dose dexamethasone versus lenalidomide plus low-dose dexamethasone as initial therapy for newly diagnosed multiple myeloma: an open-label randomised controlled trial. Lancet Oncol 11:29–37
4. Harousseau JL, Attal M, Leleu X et al (2006) Bortezomib plus dexamethasone as induction treatment prior to autologous stem cell transplantation in patients with newly diagnosed multiple myeloma: results of an IFM phase II study. Haematologica 91:1498–1505
5. San Miguel JF, Schlag R, Khuageva NK et al (2008) Bortezomib plus melphalan and prednisone for initial treatment of multiple myeloma. N Engl J Med 359:906–917
6. Reeder CB, Reece DE, Kukreti V et al (2009) Cyclophosphamide, bortezomib and dexamethasone induction for newly diagnosed multiple myeloma: high response rates in a phase II clinical trial. Leukemia 23:1337–1341
7. Richardson PG, Weller E, Lonial S et al (2010) Lenalidomide, bortezomib, and dexamethasone combination therapy in patients with newly diagnosed multiple myeloma. Blood 116:679–686
8. Kumar SK, Rajkumar SV, Dispenzieri A et al (2008) Improved survival in multiple myeloma and the impact of novel therapies. Blood 111:2516–2520
9. Kastritis E, Zervas K, Symeonidis A et al (2009) Improved survival of patients with multiple myeloma after the introduction of novel agents and the applicability of the International Staging System (ISS): an analysis of the Greek Myeloma Study Group (GMSG). Leukemia 23:1152–1157
10. Singhal S, Mehta J, Desikan R et al (1999) Antitumor activity of thalidomide in refractory multiple myeloma. N Engl J Med 341:1565–1571
11. Richardson PG, Barlogie B, Berenson J et al (2003) A phase 2 study of bortezomib in relapsed, refractory myeloma. N Engl J Med 348:2609–2617
12. Richardson PG, Sonneveld P, Schuster MW et al (2005) Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. N Engl J Med 352:2487–2498

13. International Myeloma Working Group (2003) Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the International Myeloma Working Group. Br J Haematol 121:749–757
14. Katzmann JA, Dispenzieri A, Kyle RA et al (2006) Elimination of the need for urine studies in the screening algorithm for monoclonal gammopathies by using serum immunofixation and free light chain assays. Mayo Clin Proc 81:1575–1578
15. Durie BG, Kyle RA, Belch A et al (2003) Myeloma management guidelines: a consensus report from the Scientific Advisors of the International Myeloma Foundation. Hematol J 4:379–398
16. Durie BG, Harousseau JL, Miguel JS et al (2006) International uniform response criteria for multiple myeloma. Leukemia 20:1467–1473
17. Kyle RA, Rajkumar SV (2009) Criteria for diagnosis, staging, risk stratification and response assessment of multiple myeloma. Leukemia 23:3–9
18. Birgegard G, Gascon P, Ludwig H (2006) Evaluation of anaemia in patients with multiple myeloma and lymphoma: findings of the European CANCER ANAEMIA SURVEY. Eur J Haematol 77:378–386
19. Kyle RA, Gertz MA, Witzig TE et al (2003) Review of 1027 patients with newly diagnosed multiple myeloma. Mayo Clin Proc 78:21–33
20. Eleutherakis-Papaiakovou V, Bamias A, Gika D et al (2007) Renal failure in multiple myeloma: incidence, correlations, and prognostic significance. Leuk Lymphoma 48: 337–341
21. Kyle RA, Therneau TM, Rajkumar SV et al (2002) A long-term study of prognosis in monoclonal gammopathy of undetermined significance. N Engl J Med 346:564–569
22. Kyle RA, Therneau TM, Rajkumar SV et al (2006) Prevalence of monoclonal gammopathy of undetermined significance. N Engl J Med 354:1362–1369
23. Dispenzieri A, Katzmann JA, Kyle RA et al (2010) Prevalence and risk of progression of light-chain monoclonal gammopathy of undetermined significance: a retrospective population-based cohort study. Lancet 375:1721–1728
24. Dispenzieri A, Kyle RA, Katzmann JA et al (2008) Immunoglobulin free light chain ratio is an independent risk factor for progression of smoldering (asymptomatic) multiple myeloma. Blood 111:785–789
25. Rajkumar SV, Kyle RA, Therneau TM et al (2005) Serum free light chain ratio is an independent risk factor for progression in monoclonal gammopathy of undetermined significance. Blood 106:812–817
26. Kyle RA, Rajkumar SV (2004) Multiple myeloma. N Engl J Med 351:1860–1873
27. Landgren O, Katzmann JA, Hsing AW et al (2007) Prevalence of monoclonal gammopathy of undetermined significance among men in Ghana. Mayo Clin Proc 82:1468–1473
28. Landgren O, Kyle RA, Pfeiffer RM et al (2009) Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: a prospective study. Blood 113:5412–5417
29. Weiss BM, Abadie J, Verma P, Howard RS, Kuehl WM (2009) A monoclonal gammopathy precedes multiple myeloma in most patients. Blood 113:5418–5422
30. Smadja NV, Fruchart C, Isnard F et al (1998) Chromosomal analysis in multiple myeloma: cytogenetic evidence of two different diseases. Leukemia 12:960–969
31. Debes-Marun CS, Dewald GW, Bryant S et al (2003) Chromosome abnormalities clustering and its implications for pathogenesis and prognosis in myeloma. Leukemia 17:427–436
32. Fonseca R, Debes-Marun CS, Picken EB et al (2003) The recurrent IgH translocations are highly associated with nonhyperdiploid variant multiple myeloma. Blood 102:2562–2567
33. Drach J, Schuster J, Nowotny H et al (1995) Multiple myeloma: high incidence of chromosomal aneuploidy as detected by interphase fluorescence in situ hybridization. Cancer Res 55:3854–3859
34. Perez-Simon JA, Garcia-Sanz R, Tabernero MD et al (1998) Prognostic value of numerical chromosome aberrations in multiple myeloma: a FISH analysis of 15 different chromosomes. Blood 91:3366–3371

35. Garcia-Sanz R, Orfao A, Gonzalez M et al (1995) Prognostic implications of DNA aneuploidy in 156 untreated multiple myeloma patients. Castelano-Leones (Spain) Cooperative Group for the Study of Monoclonal Gammopathies. Br J Haematol 90:106–112
36. Wuilleme S, Robillard N, Lode L et al (2005) Ploidy, as detected by fluorescence in situ hybridization, defines different subgroups in multiple myeloma. Leukemia 19:275–278
37. Chng WJ, Van Wier SA, Ahmann GJ et al (2005) A validated FISH trisomy index demonstrates the hyperdiploid and nonhyperdiploid dichotomy in MGUS. Blood 106:2156–2161
38. Drach J, Angerler J, Schuster J et al (1995) Interphase fluorescence in situ hybridization identifies chromosomal abnormalities in plasma cells from patients with monoclonal gammopathy of undetermined significance. Blood 86:3915–3921
39. Fonseca R, Bailey RJ, Ahmann GJ et al (2002) Genomic abnormalities in monoclonal gammopathy of undetermined significance. Blood 100:1417–1424
40. Carrasco DR, Tonon G, Huang Y et al (2006) High-resolution genomic profiles define distinct clinico-pathogenetic subgroups of multiple myeloma patients. Cancer Cell 9:313–325
41. Chng WJ, Santana-Davila R, Van Wier SA et al (2006) Prognostic factors for hyperdiploid-myeloma: effects of chromosome 13 deletions and IgH translocations. Leukemia 20:807–813
42. Fonseca R, Blood E, Rue M et al (2003) Clinical and biologic implications of recurrent genomic aberrations in myeloma. Blood 101:4569–4575
43. Bergsagel PL, Kuehl WM (2001) Chromosome translocations in multiple myeloma. Oncogene 20:5611–5622
44. Bergsagel PL, Kuehl WM (2005) Molecular pathogenesis and a consequent classification of multiple myeloma. J Clin Oncol 23:6333–6338
45. Fonseca R, Barlogie B, Bataille R et al (2004) Genetics and cytogenetics of multiple myeloma: a workshop report. Cancer Res 64:1546–1558
46. Avet-Loiseau H, Attal M, Moreau P et al (2007) Genetic abnormalities and survival in multiple myeloma: the experience of the Intergroupe Francophone du Myelome. Blood 109:3489–3495
47. Lopez-Corral L, Gutierrez NC, Vidriales MB et al (2011) The progression from MGUS to smoldering myeloma and eventually to multiple myeloma involves a clonal expansion of genetically abnormal plasma cells. Clin Cancer Res 17:1692–1700
48. Avet-Loiseau H, Facon T, Grosbois B et al (2002) Oncogenesis of multiple myeloma: 14q32 and 13q chromosomal abnormalities are not randomly distributed, but correlate with natural history, immunological features, and clinical presentation. Blood 99:2185–2191
49. Fonseca R, Blood EA, Oken MM et al (2002) Myeloma and the t(11;14)(q13;q32); evidence for a biologically defined unique subset of patients. Blood 99:3735–3741
50. Gertz MA, Lacy MQ, Dispenzieri A et al (2005) Clinical implications of t(11;14)(q13;q32), t(4;14)(p16.3;q32), and -17p13 in myeloma patients treated with high-dose therapy. Blood 106:2837–2840
51. Moreau P, Facon T, Leleu X et al (2002) Recurrent 14q32 translocations determine the prognosis of multiple myeloma, especially in patients receiving intensive chemotherapy. Blood 100:1579–1583
52. Hoyer JD, Hanson CA, Fonseca R, Greipp PR, Dewald GW, Kurtin PJ (2000) The (11;14)(q13;q32) translocation in multiple myeloma. A morphologic and immunohistochemical study. Am J Clin Pathol 113:831–837
53. Garand R, Avet-Loiseau H, Accard F, Moreau P, Harousseau JL, Bataille R (2003) t(11;14) and t(4;14) translocations correlated with mature lymphoplasmacytoid and immature morphology, respectively, in multiple myeloma. Leukemia 17:2032–2035
54. Fonseca R, Witzig TE, Gertz MA et al (1998) Multiple myeloma and the translocation t(11;14)(q13;q32): a report on 13 cases. Br J Haematol 101:296–301
55. Hayman SR, Bailey RJ, Jalal SM et al (2001) Translocations involving the immunoglobulin heavy-chain locus are possible early genetic events in patients with primary systemic amyloidosis. Blood 98:2266–2268
56. Chiecchio L, Protheroe RK, Ibrahim AH et al (2006) Deletion of chromosome 13 detected by conventional cytogenetics is a critical prognostic factor in myeloma. Leukemia 20: 1610–1617

57. Keats JJ, Reiman T, Maxwell CA et al (2003) In multiple myeloma, t(4;14)(p16;q32) is an adverse prognostic factor irrespective of FGFR3 expression. Blood 101:1520–1529
58. Chang H, Qi XY, Samiee S et al (2005) Genetic risk identifies multiple myeloma patients who do not benefit from autologous stem cell transplantation. Bone Marrow Transplant 36:793–796
59. Chang H, Sloan S, Li D et al (2004) The t(4;14) is associated with poor prognosis in myeloma patients undergoing autologous stem cell transplant. Br J Haematol 125:64–68
60. Avet-Loiseau H, Soulier J, Fermand JP et al (2010) Impact of high-risk cytogenetics and prior therapy on outcomes in patients with advanced relapsed or refractory multiple myeloma treated with lenalidomide plus dexamethasone. Leukemia 24:623–628
61. Trudel S, Ely S, Farooqi Y et al (2004) Inhibition of fibroblast growth factor receptor 3 induces differentiation and apoptosis in t(4;14) myeloma. Blood 103:3521–3528
62. Trudel S, Li ZH, Wei E et al (2005) CHIR-258, a novel, multitargeted tyrosine kinase inhibitor for the potential treatment of t(4;14) multiple myeloma. Blood 105:2941–2948
63. Chesi M, Bergsagel PL, Shonukan OO et al (1998) Frequent dysregulation of the c-maf proto-oncogene at 16q23 by translocation to an Ig locus in multiple myeloma. Blood 91:4457–4463
64. Shaughnessy JD Jr, Zhan F, Burington BE et al (2007) A validated gene expression model of high-risk multiple myeloma is defined by deregulated expression of genes mapping to chromosome 1. Blood 109:2276–2284
65. Ross FM, Chiecchio L, Dagrada G et al (2010) The t(14;20) is a poor prognostic factor in myeloma but is associated with long-term stable disease in monoclonal gammopathies of undetermined significance. Haematologica 95:1221–1225
66. Avet-Loiseau H, Malard F, Campion L et al (2011) Translocation t(14;16) and multiple myeloma: is it really an independent prognostic factor? Blood 117:2009–2011
67. Albarracin F, Fonseca R (2011) Plasma cell leukemia. Blood Rev 25:107–112
68. Hayman SR, Fonseca R (2001) Plasma cell leukemia. Curr Treat Options Oncol 2:205–216
69. Fonseca R, Oken MM, Harrington D et al (2001) Deletions of chromosome 13 in multiple myeloma identified by interphase FISH usually denote large deletions of the q arm or monosomy. Leukemia 15:981–986
70. Avet-Louseau H, Daviet A, Sauner S, Bataille R (2000) Chromosome 13 abnormalities in multiple myeloma are mostly monosomy 13. Br J Haematol 111:1116–1117
71. Facon T, Avet-Loiseau H, Guillerm G et al (2001) Chromosome 13 abnormalities identified by FISH analysis and serum beta2-microglobulin produce a powerful myeloma staging system for patients receiving high-dose therapy. Blood 97:1566–1571
72. Desikan R, Barlogie B, Sawyer J et al (2000) Results of high-dose therapy for 1000 patients with multiple myeloma: durable complete remissions and superior survival in the absence of chromosome 13 abnormalities. Blood 95:4008–4010
73. Zojer N, Konigsberg R, Ackermann J et al (2000) Deletion of 13q14 remains an independent adverse prognostic variable in multiple myeloma despite its frequent detection by interphase fluorescence in situ hybridization. Blood 95:1925–1930
74. Fonseca R, Harrington D, Oken MM et al (2002) Biological and prognostic significance of interphase fluorescence in situ hybridization detection of chromosome 13 abnormalities (delta13) in multiple myeloma: an eastern cooperative oncology group study. Cancer Res 62:715–720
75. Drach J, Ackermann J, Fritz E et al (1998) Presence of a p53 gene deletion in patients with multiple myeloma predicts for short survival after conventional-dose chemotherapy. Blood 92:802–809
76. Tiedemann RE, Gonzalez-Paz N, Kyle RA et al (2008) Genetic aberrations and survival in plasma cell leukemia. Leukemia 22:1044–1052
77. Mazars GR, Portier M, Zhang XG et al (1992) Mutations of the p53 gene in human myeloma cell lines. Oncogene 7:1015–1018
78. Sawyer JR, Tricot G, Mattox S, Jagannath S, Barlogie B (1998) Jumping translocations of chromosome 1q in multiple myeloma: evidence for a mechanism involving decondensation of pericentromeric heterochromatin. Blood 91:1732–1741

79. Zhan F, Colla S, Wu X et al (2007) CKS1B, over expressed in aggressive disease, regulates multiple myeloma growth and survival through SKP2- and p27Kip1-dependent and independent mechanisms. Blood 109(11):4995–5001
80. Hanamura I, Stewart JP, Huang Y et al (2006) Frequent gain of chromosome band 1q21 in plasma-cell dyscrasias detected by fluorescence in situ hybridization: incidence increases from MGUS to relapsed myeloma and is related to prognosis and disease progression following tandem stem-cell transplantation. Blood 108:1724–1732
81. Chesi M, Brents LA, Ely SA et al (2001) Activated fibroblast growth factor receptor 3 is an oncogene that contributes to tumor progression in multiple myeloma. Blood 97:729–736
82. Liu P, Leong T, Quam L et al (1996) Activating mutations of N- and K-ras in multiple myeloma show different clinical associations: analysis of the Eastern Cooperative Oncology Group Phase III Trial. Blood 88:2699–2706
83. Bezieau S, Devilder MC, Avet-Loiseau H et al (2001) High incidence of N and K-Ras activating mutations in multiple myeloma and primary plasma cell leukemia at diagnosis. Hum Mutat 18:212–224
84. Bergsagel PL, Kuehl WM, Zhan F, Sawyer J, Barlogie B, Shaughnessy J Jr (2005) Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. Blood 106:296–303
85. Chng WJ, Braggio E, Mulligan G et al (2008) The centrosome index is a powerful prognostic marker in myeloma and identifies a cohort of patients that might benefit from aurora kinase inhibition. Blood 111:1603–1609
86. Rawstron AC, Orfao A, Beksac M et al (2008) Report of the European Myeloma Network on multiparametric flow cytometry in multiple myeloma and related disorders. Haematologica 93:431–438
87. Paiva B, Vidriales MB, Perez JJ et al (2009) Multiparameter flow cytometry quantification of bone marrow plasma cells at diagnosis provides more prognostic information than morphological assessment in myeloma patients. Haematologica 94:1599–1602
88. Chapman MA, Lawrence MS, Keats JJ et al (2011) Initial genome sequencing and analysis of multiple myeloma. Nature 471:467–472
89. Seidl S, Ackermann J, Kaufmann H et al (2004) DNA-methylation analysis identifies the E-cadherin gene as a potential marker of disease progression in patients with monoclonal gammopathies. Cancer 100:2598–2606
90. Gonzalez-Paz N, Chng WJ, McClure RF et al (2007) Tumor suppressor p16 methylation in multiple myeloma: biological and clinical implications. Blood 109:1228–1232
91. Galm O, Wilop S, Reichelt J et al (2004) DNA methylation changes in multiple myeloma. Leukemia 18:1687–1692
92. Chim CS, Kwong YL, Fung TK, Liang R (2004) Methylation profiling in multiple myeloma. Leuk Res 28:379–385
93. Rossi D, Capello D, Gloghini A et al (2004) Aberrant promoter methylation of multiple genes throughout the clinico-pathologic spectrum of B-cell neoplasia. Haematologica 89:154–164
94. Mateos MV, Garcia-Sanz R, Lopez-Perez R et al (2002) Methylation is an inactivating mechanism of the p16 gene in multiple myeloma associated with high plasma cell proliferation and short survival. Br J Haematol 118:1034–1040
95. Guillerm G, Depil S, Wolowiec D, Quesnel B (2003) Different prognostic values of p15(INK4b) and p16(INK4a) gene methylations in multiple myeloma. Haematologica 88:476–478
96. Chim CS, Pang R, Fung TK, Choi CL, Liang R (2007) Epigenetic dysregulation of Wnt signaling pathway in multiple myeloma. Leukemia 21:2527–2536
97. Walker BA, Wardell CP, Chiecchio L et al (2011) Aberrant global methylation patterns affect the molecular pathogenesis and prognosis of multiple myeloma. Blood 117:553–562
98. Salhia B, Baker A, Ahmann G, Auclair D, Fonseca R, Carpten J (2010) DNA methylation analysis determines the high frequency of genic hypomethylation and low frequency of hypermethylation events in plasma cell tumors. Cancer Res 70:6934–6944

99. Keats J, Chesi M, Egan JB et al (2012) Clonal competition with alternating dominance in multiple myeloma. Blood 120:1067–1076
100. Egan JB, Shi CX, Tembe W et al (2012) Whole Genome Sequencing of Multiple Myeloma From Diagnosis to Plasma Cell Leukemia Reveals Genomic Initiating Events, Evolution and Clonal Tides. Blood 120:1060–1066
101. Walker BA, Wardell CP, Melchor L et al (2012) Intraclonal heterogeneity and distinct molecular mechanisms characterize the development of t(4;14) and t(11;14) myeloma. Blood 120:1077–1086
102. Navin N, Kendall J, Troge J et al (2011) Tumour evolution inferred by single-cell sequencing. Nature 472:90–94
103. Anderson K, Lutz C, van Delft FW et al (2011) Genetic variegation of clonal architecture and propagating cells in leukaemia. Nature 469:356–361
104. Avet-Loiseau H, Facon T, Daviet A et al (1999) 14q32 translocations and monosomy 13 observed in monoclonal gammopathy of undetermined significance delineate a multistep process for the oncogenesis of multiple myeloma. Intergroupe Francophone du Myelome. Cancer Res 59:4546–4550
105. Chiecchio L, Dagrada GP, Ibrahim AH et al (2009) Timing of acquisition of deletion 13 in plasma cell dyscrasias is dependent on genetic context. Haematologica 94:1708–1713
106. Kuehl WM, Bergsagel PL (2002) Multiple myeloma: evolving genetic events and host interactions. Nat Rev Cancer 2:175–187
107. Smadja NV, Leroux D, Soulier J et al (2003) Further cytogenetic characterization of multiple myeloma confirms that 14q32 translocations are a very rare event in hyperdiploid cases. Genes Chromosomes Cancer 38:234–239
108. Konigsberg R, Ackermann J, Kaufmann H et al (2000) Deletions of chromosome 13q in monoclonal gammopathy of undetermined significance. Leukemia 14:1975–1979
109. Zandecki M, Obein V, Bernardi F et al (1995) Monoclonal gammopathy of undetermined significance: chromosome changes are a common finding within bone marrow plasma cells. Br J Haematol 90:693–696
110. Brousseau M, Leleu X, Gerard J et al (2007) Hyperdiploidy is a common finding in monoclonal gammopathy of undetermined significance and monosomy 13 is restricted to these hyperdiploid patients. Clin Cancer Res 13:6026–6031
111. Ackermann J, Meidlinger P, Zojer N et al (1998) Absence of p53 deletions in bone marrow plasma cells of patients with monoclonal gammopathy of undetermined significance. Br J Haematol 103:1161–1163
112. Corradini P, Inghirami G, Astolfi M et al (1994) Inactivation of tumor suppressor genes, p53 and Rb1, in plasma cell dyscrasias. Leukemia 8:758–767
113. Rosinol L, Blade J, Esteve J et al (2003) Smoldering multiple myeloma: natural history and recognition of an evolving type. Br J Haematol 123:631–636
114. Chiecchio L, Dagrada GP, Protheroe RK et al (2009) Loss of 1p and rearrangement of MYC are associated with progression of smouldering myeloma to myeloma: sequential analysis of a single case. Haematologica 94:1024–1028
115. Shou Y, Martelli ML, Gabrea A et al (2000) Diverse karyotypic abnormalities of the c-myc locus associated with c-myc dysregulation and tumor progression in multiple myeloma. Proc Natl Acad Sci USA 97:228–233
116. Avet-Loiseau H, Gerson F, Magrangeas F, Minvielle S, Harousseau JL, Bataille R (2001) Rearrangements of the c-myc oncogene are present in 15% of primary human multiple myeloma tumors. Blood 98:3082–3086
117. Chesi M, Robbiani DF, Sebag M et al (2008) AID-dependent activation of a MYC transgene induces multiple myeloma in a conditional mouse model of post-germinal center malignancies. Cancer Cell 13:167–180
118. Rasmussen T, Kuehl M, Lodahl M, Johnsen HE, Dahl IM (2005) Possible roles for activating RAS mutations in the MGUS to MM transition and in the intramedullary to extramedullary transition in some plasma cell tumors. Blood 105:317–323

119. Chng WJ, Gonzalez-Paz N, Price-Troska T et al (2008) Clinical and biological significance of RAS mutations in multiple myeloma. Leukemia 22:2280–2284
120. Keats JJ, Fonseca R, Chesi M et al (2007) Promiscuous mutations activate the noncanonical NF-kappaB pathway in multiple myeloma. Cancer Cell 12:131–144
121. Annunziata CM, Davis RE, Demchenko Y et al (2007) Frequent engagement of the classical and alternative NF-kappaB pathways by diverse genetic abnormalities in multiple myeloma. Cancer Cell 12:115–130

Chapter 2
Prognostic Implication of Genetic Changes (Cytogenetics, and FISH, Gains and Losses of DNA by SNP Array and aCGH) in Risk Stratification in Myeloma

Jill Corre and Hervé Avet-Loiseau

Abstract Multiple myeloma (MM) is characterized by a huge heterogeneity in survival. Most of these differences can be captured by the variability of genetic events occurring within the malignant plasma cells. At the chromosomal level, the two most important changes are the del(17p) and the translocation t(4;14), both associated with a poor outcome. Recent data using modern genomics, such as gene expression profiling, or SNParray, revealed another level of complexity, which can be utilized for a better prognostic assessment. However, these techniques are still research tools. Whether there will be routine techniques in the future is an open question.

As for other hematopoietic malignancies, and especially acute leukemias, chromosomal abnormalities have been shown to represent very strong predictors of evolution in multiple myeloma. However, and in contrast with acute leukemias, the use of genetics to predict patient evolution, and thus to adapt therapy to this risk, is only in its infancy. This gap between myeloma and leukemias is especially related to technical pitfalls (see next section). With the development of novel technologies and the systematization of genetic analyses in the diagnostic evaluation of the patients, there is no doubt that genetics will take a major place in the management of patients with myeloma. The goal of this chapter is to summarize our current knowledge of chromosomal abnormalities in myeloma as well as to show their role in the oncogenesis, their impact on the natural history of the disease, and their potential utility in patient management.

J. Corre, Pharm.D. • H. Avet-Loiseau, M.D., Ph.D. (✉)
Unit for Genomics of Myeloma,
CHU Rangueil, TSA 50032, 31059 Toulouse Cedex 9, France
e-mail: avet-loiseau.h@chu-toulouse.fr

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_2,

2.1 Definitions and Technical Aspects

Classically, the landscape of the chromosomal abnormalities observed in a specific disease is obtained through the analysis of hundreds of patients using classical cytogenetics. In myeloma, this approach has been less successful than in leukemias because of the difficulty to generate metaphases within the tumor clone. Recent studies using high-density CGH (comparative genomic hybridization) or SNP (single-nucleotide polymorphism) arrays have shown that virtually 100% of the patients with myeloma display chromosomal abnormalities [1, 2]. In contrast, cytogenetic analyses reported in the literature have shown that an abnormal karyotype is identified in <30% of the patients [3–8]. This discrepancy is not related to the resolution differences between the two techniques, since many of the genetic changes identified by CGH involved large chromosomal regions. The two major explanations are related to the low proliferation of malignant plasma cells and to the usual low percentage of plasma cells within the biological specimens sent to the cytogenetic laboratories. Thus, normal karyotypes in myeloma should not be interpreted as the absence of chromosomal changes within the tumor clone, but as the result of the division of normal myeloid bone marrow cells, representing the constitutional DNA.

This failure to obtain informative karyotypes has led researchers to use other techniques, not dependent upon the generation of clonal metaphases, especially fluorescence in situ hybridization (FISH) on interphase cells [9–12]. This technique enables to assess the presence or not of specific chromosomal changes in every patient with myeloma, whatever his/her in vitro proliferation potential. However, because of the common low plasma cell percentage within the bone marrow specimens sent to the lab (median = 6% in the IFM experience), the technique cannot be performed straightforward, as usual in other hematopoietic malignancies. The plasma cells have to be selected, either by a previous cell sorting or by the concomitant labeling of the cytoplasmic Ig light chains, thereby enabling an unambiguous identification of the plasma cells. Both strategies are equivalent: cell sorting enabling to perform further analyses, like gene expression profiling or copy number analyses on pure plasma cell populations.

2.2 Specific Chromosomal Changes

2.2.1 Aneuploidy

Despite the scarcity of large cytogenetic studies, the analysis of the literature reveals that abnormal karyotypes can be separated in two groups, almost identical in numbers: patients with a hyperdiploid karyotype (i.e., with more than 46 chromosomes), and those with a hypo-or pseudodiploid one (i.e., with less than 46 chromosomes, or 46 chromosomes with structural aberrations). Although this classification (hyperdiploidy vs. non-hyperdiploidy) is somewhat artificial, hyperdiploidy appears as a relatively homogeneous group. Most of the patients in this category present with a

high number of chromosomes (median = 54), involving nonrandom gains. Actually, trisomies involve especially odd chromosomes, mostly chromosomes 3, 5, 7, 9, 11, 15, 19, and 21. In contrast, non-hyperdiploid karyotypes are much more heterogeneous, even though some chromosome losses and some structural aberrations appear also nonrandom. The most recurrent ones are monosomy 13, 8p deletions, 1q gains, and 14q32 translocations. Nevertheless, these abnormalities are not specific of the non-hyperdiploid category and can be observed in hyperdiploid karyotypes. Of note, most (if not all) of the human myeloma cell lines derived from patient specimens belong to the non-hyperdiploid category. This bias has to be considered when cell lines are used as models of the human disease.

Few studies have analyzed the prognostic value of this classification [8, 13–15]. Hyperdiploidy seems to be associated with a better prognosis. However, these analyses are hampered by several methodological biases, including the retrospective nature of the analyses, the heterogeneity in the patient population, and the disparity of the treatment strategies. More recently, a few studies have used interphase FISH approaches to define ploidy [16, 17]. These studies did confirm the better prognosis of hyperdiploidy, but it has to be demonstrated that this prognostic value is not dependent of other confounding parameters. In the IFM experience, hyperdiploidy was not an independent prognostic factor but was associated with a lower incidence of del(13), t(4;14), and del(17p) (see below, [18]).

2.2.2 Chromosome 13 Abnormalities

Chromosome 13 is frequently abnormal in myeloma. Most of the abnormalities are monosomies and, less frequently, translocations or interstitial deletions are observed, usually involving the 13q14 region. In cytogenetic series, chromosome 13 abnormalities, or del(13), are observed in about half of the abnormal karyotypes. This frequency has been confirmed in interphase FISH studies [19–23]. The role of del(13) in the oncogenesis of myeloma is still a matter of debate. The abnormality is probably an early event (and even possibly a primary genetic event), since it is observed with similar frequencies in premalignant MGUS stages and in patients at relapse. The molecular consequences of these chromosomal losses are almost unknown, although a molecular signature has been identified using expression profiling [24, 25]. However, because most of del(13) are in fact monosomies, many genes are lost and may be deregulated. The quest for the Holy Grail is still opened!

The first recognition of the negative impact of del(13) on survival came from Little Rock in 1995, based on cytogenetic data [26]. Later on, several groups demonstrated that this poor-prognosis feature was retained when the del(13) was identified by interphase FISH. However, a debate still exists regarding the prognostic value of del(13) depending on the technique used. Few reports have compared the prognostic value of both techniques on the same patients. However, those reports are concordant to show that del(13) identified by interphase FISH only (with a concomitant normal karyotype) does predict for a shorter survival

than those patients lacking del(13) [27, 28]. In the recent IFM study, del(13) identified by FISH was not an independent prognostic factor, since its association with a poor prognosis is related to the concomitant t(4;14) or del(17p) (see below) [18]. Actually, del(13) per se may not confer a specific prognosis in myeloma and may be considered as a marker frequently associated with other more specific poor-prognosis factors.

2.2.3 Translocations Involving the 14q32 Region

Cytogenetic analyses did identify the chromosomal 14q32 region as a recurrent hotspot of translocations in myeloma, with a frequency of about 30%. In most cases, these translocations identified on the karyotype were t(11;14)(q13;q32) [29, 30]. The interest for this region grew in 1997 with the publication by Bergsagel et al. showing that at least 90% of the human myeloma cell lines did present an illegitimate molecular rearrangement of the *IGH* gene, located at 14q32 [31]. Interestingly, some of the cell lines displaying a rearrangement by Southern blot looked normal at karyotype, leading to hypothesize cryptic rearrangements. This hypothesis has been demonstrated by several authors, using different techniques. Actually, the analysis of primary tumors from patients showed that a 14q32 translocation was present in about 60% of the patients [12, 13, 32, 33]. Furthermore, it has been shown that these 14q32 translocations did involve several chromosomal partners, and that some of these translocations were karyotypically silent, explaining, at least in part, the frequent discrepancy between FISH and cytogenetics. However, the picture is different than that observed in some non-Hodgkin lymphomas, in which a unique, typical 14q32 translocation is the hallmark of a lymphoma subtype. In myeloma, at least 30 different chromosomal regions have been involved in translocations with the 14q32 region. Despite this heterogeneity, a few recurrent specific translocations have been described: the t(11;14)(q13;q32) in ~20% of the patients, the t(4;14)(p16;q32) in ~15% of the patients, and the t(14;16)(q32;q23) in ~5% of the patients. These more frequent translocations may actually define myeloma subtypes.

The **t(11;14)(q13;q32)** is identical to that observed in mantle cell lymphomas. The breakpoints involve the *IGH* gene at 14q32, and the *CCND1* gene at 11q13, encoding the cyclin D1 protein [34, 35]. One of the molecular consequences of the translocation is the upregulation of cyclin D1. So far, the oncogenic role of the translocation is unknown. Although the cyclin D1 has been involved in the activation of proliferation, the t(11;14) myelomas are characterized by a low proliferative index and a frequent morphology of small mature plasma cells [36, 37]. They are more likely to express the CD20 at the cell surface [38]. Clinically, this type of myeloma is not remarkable. Even though preliminary reports did show on a better survival [39], more recent and larger studies did not confirm this prognostic impact [18, 40].

The **t(4;14)(p16;q32)** is so far specific of myeloma and has never been described in other malignancies. However, this specificity has to be taken with caution since

the t(4;14) is "cryptic," meaning that it is not detectable by the karyotype. The molecular cloning of the translocation revealed a peculiar situation so far unique in hematology [41–43]. The translocation leads to the deregulation of two genes located at 4p16. The *FGFR3* gene, which encodes a receptor for the fibroblast growth factors, is located on the telomeric side of the breakpoints. The translocation displaces the *FGFR3* gene to the 14q32 region, leading to the molecular activation of the gene transcription. Because FGFR3 has a tyrosine kinase activity, it is a good candidate for an oncogenic function. Several tyrosine kinase inhibitors are currently tested in order to inhibit this function. However, even though in vitro and animal models did favor this hypothesis [44, 45], several reports did show that about one-third of the patients with t(4;14) did not display FGFR3 overexpression [46, 47]. This observation supports the hypothesis of another molecular consequence of the translocation. Actually, the translocation disrupts another gene located at 4p16, a gene identified with the cloning of t(4;14) translocations. This gene has been named *MMSET* (for *Multiple Myeloma SET domain* gene), because of some degree of homology with other genes containing a *SET* domain, like *MLL*. The translocation disrupts the *MMSET* gene within the first introns, leading to the generation of a novel chimeric *IGH-MMSET* gene. So far, neither the physiological function of MMSET (it could be involved in the regulation of chromatin remodeling), nor the consequences of its deregulation by the translocation are known. However, this chimeric gene is constantly present in the t(4;14) cases, and *MMSET* may represent the primary target of the t(4;14).

Several studies did show that the t(4;14) is associated with a poor prognosis (Fig. 2.1). However, recent large-scale studies did suggest that all the patients with t(4;14) do not present a very short survival and that other factors may have an influence on outcome [18]. For instance, the IFM did show that patients with t(4;14) and a low β2-microglobulin level may enjoy longer survivals than those presenting the translocation with a high β2-microglobulin level [48]. Recent data suggest that the poor prognosis associated with the t(4;14) might be (at least partially) overcome by novel therapies, especially bortezomib-based combinations [49]. Interestingly, genetic studies using FISH did show that at least 85% of the patients with t(4;14) do also present del(13). The reasons for this strong association are so far unknown, but clinically it appears that patients displaying the two genetic abnormalities have a poorer prognosis than those lacking the del(13). Finally, it has to be highlighted that the frequency of the t(4;14) is higher in human myeloma cell lines than in the patients (25 vs. 15%). This discrepancy may reflect the intrinsic aggressiveness conferred by the translocation, possibly facilitating the generation of cell lines.

The **t(14;16)(q32;q23)** is also specific for myeloma. The cloning of the translocation did show that the 16q23 breakpoints occur in the vicinity of the *MAF* gene, leading to its translocation on the derivative chromosome 14, and finally to its overexpression [50]. Further investigations did show that MAF is a transcription factor that positively regulates other genes like *CCND2* or *ITGB7* [51]. The translocation is rare in myeloma (~3% of the patients), whereas it is frequently observed in myeloma cell lines (~25%). Here again, the discrepancy in frequencies between patients and cell lines might be related to the aggressiveness conferred by the t(14;16). Actually, very

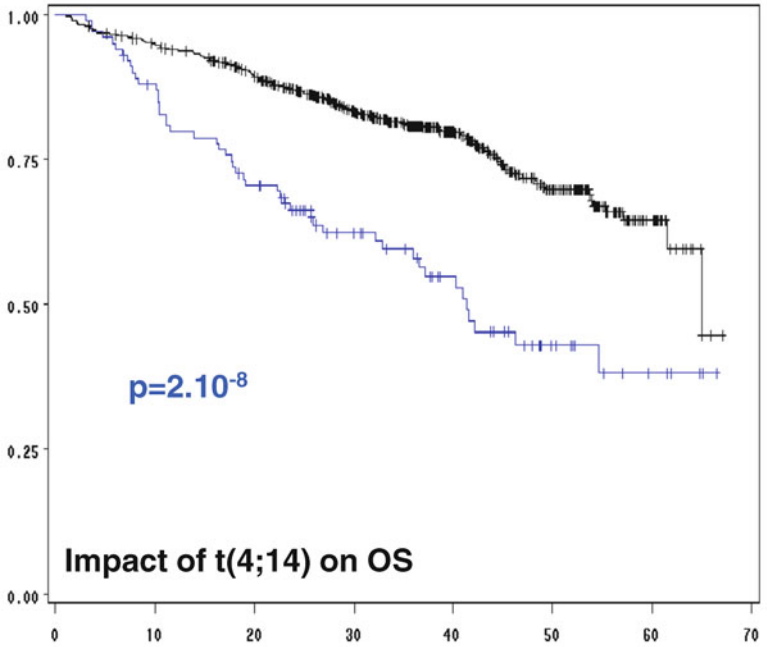


Fig. 2.1 Overall survival for patients presenting a t(4;14) or not. *Black* represents patients without and *blue* with t(4;14). This research was originally published in *Blood*. Ref 18 © the American Society of Hematology

few reports have analyzed the prognostic impact of this translocation. The rare publications on this topic did report a shorter survival for patients presenting the translocation, which is almost always associated with del(13). This translocation has to be related to another 14q32 translocation, the **t(14;20)(q32;q11)** observed in only a few percentage of patients [52]. This latter translocation deregulates *MAFB*, a gene belonging to the *MAF* family, with molecular consequences so far unknown.

Finally, almost 20% of the patients display a 14q32 translocation with other multiple chromosomal partners. The role of these nonrecurrent translocations is totally unknown. In contrast to the recurrent 14q32 translocations described above, these ones are mostly observed in patients with hyperdiploid karyotypes. They may reflect a genetic instability, especially focused on the 14q32 region and the *IGH* gene, which is physiologically rearranged at several stages of the B-cell differentiation.

2.2.4 Deletions 17p

More recently, a loss of the short arm of the chromosome 17, i.e., del(17p), has been described in about 10% of the patients with myeloma [53, 54]. These deletions are not specific to myeloma and have been reported in numerous tumor types, like

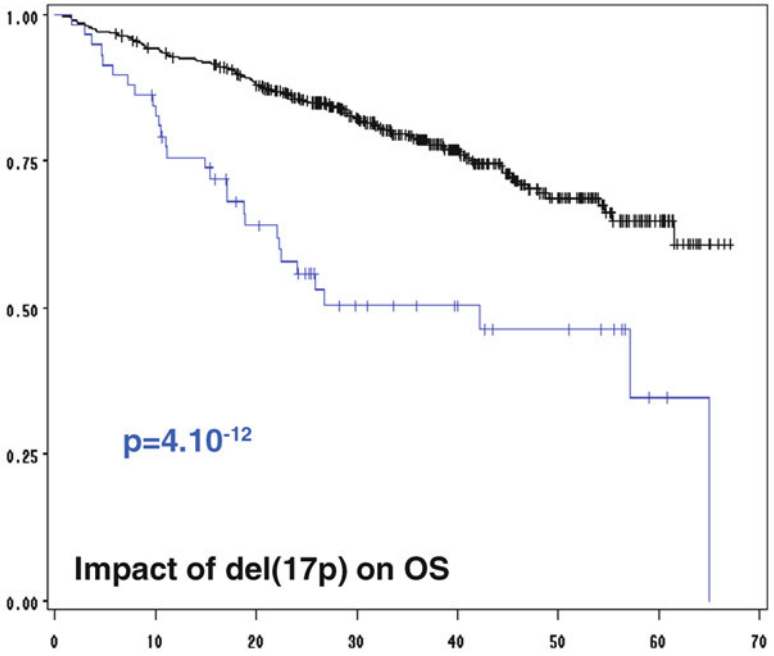


Fig. 2.2 Overall survival for patients presenting a del(17p) or not. *Black* represents patients without and *blue* with del(17p). This research was originally published in *Blood*. Ref 18 © the American Society of Hematology

chronic lymphocytic leukemia, acute myeloid leukemia, or many solid tumors. These losses generally involved the major part of the short arm of chromosome 17, thus leading to the loss of many genes, this region being particularly rich in coding sequences. However, most authors did focus on the *TP53* gene, located at 17p13, since this gene is mutated in about half of the tumor types. Nevertheless, this hypothesis would suppose a mutation of the other allele. This hypothesis is attractive since patients presenting a del(17p) usually display a poor prognosis, whatever the type of treatment (conventional or intensive). Since P53 is involved in the mechanisms of cell death induced by most chemotherapeutic agents, its invalidation may participate to the chemoresistance presented by patients with del(17p). However, this hypothesis has to be demonstrated. Mutations of the *TP53* gene are a rare event in myeloma, especially at diagnosis [55–57]. The IFM recently reported that *TP53* mutations are exclusively observed in patients with del(17p), with a frequency of around 30–40% [58]. Whatever the mechanism, these deletions are associated with a poor outcome, observed in all the studies reported so far (Fig. 2.2). In contrast to translocations involving the 14q32 region, they are thought to be secondary events and can be acquired during evolution.

2.2.5 Abnormalities of the 1q Region

More recently, the Arkansas group did report on the prognostic value of 1q gains [59]. In cytogenetic studies, extracopies of the long arm of chromosome 1 have been described in about one-third of the patients [60]. Actually, this abnormality is not restricted to myeloma and has been reported in many tumor types, both in hematological neoplasms and in solid tumors. In the Arkansas study, this abnormality came up as the strongest prognostic factor. They did show that patients with either a gain of the 1q21 chromosomal region or with overexpression of the *CKS1B* gene (located at 1q21) presented a poor outcome in the "Total Therapy" program. Since this pioneering report, several groups did confirm the poor outcome of patients with 1q gains. However, the Mayo Clinic [61] and the IFM (unpublished results) did show in independent cohorts of patients that this parameter was not retained in multivariate analyses and that its prognostic value disappeared when combined with other classical biological and genetic prognostic factors. Thus, further studies are required in order to understand the real prognostic impact of 1q gains.

2.3 Practical Use of Cytogenetic Data in Routine Practice

The first question is as follows: Should we perform chromosomal analysis for every patient and (in case of a positive answer) how? Analyzing the prognostic impact of some chromosomal changes, it is clear that cytogenetics (conventional or molecular) displays a prognostic value in myeloma, similar to other hematological neoplasms. Thus, in agreement with general hematological practice, cytogenetic analysis at diagnosis should be considered as a "good clinical practice," at least to define the prognosis of the disease in each typical patient. Furthermore, the improvement of our knowledge of myeloma biology will definitely have a major impact in the improvement of patient management, either by selecting optimal treatments for each patient or by helping in the development of novel drugs.

The way to perform chromosomal analysis is more debatable. Both conventional cytogenetics and molecular cytogenetics present their own advantages. Conventional cytogenetics allows a global envision of the chromosomal abnormalities throughout the entire genome. Furthermore, the prognostic value of a typical chromosomal abnormality will probably be stronger if detected at karyotype rather than with FISH, essentially because an abnormal karyotype is, by definition, linked to proliferation (at least in vitro). Taking into account the prognostic impact of proliferation in myeloma, karyotypic abnormalities display a strong prognostic value. However, this is not a sufficient reason to recommend to perform conventional cytogenetics. This theoretical advantage has to be faced with the frequent absence of any detectable chromosomal change on the karyotype and with the heaviness of cytogenetic assessment. Actually, karyotyping is highly time consuming, and regarding its low informativity in myeloma, it has to be seriously evaluated before it is proposed, especially in a multicenter setting. Thus, more and more cooperative groups did

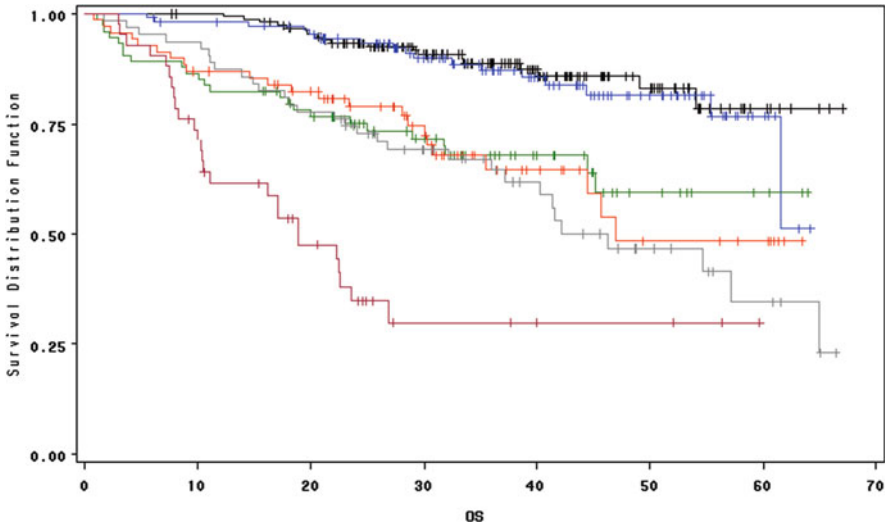


Fig. 2.3 Influence of t(4;14), del(17p), and β2-microglobulin level on overall survival. The *black curve* is for patients lacking del(13), t(4;14), and del(17p), and presenting a low β2-microglobulin level (≤ 4 mg/L). The *green curve* represents the similar patients, but with a high β2-microglobulin level (> 4 mg/L). The *blue curve* depicts patients lacking t(4;14) and del(17p) with a low β2-microglobulin level, but presenting a del(13). The *red curve* represents patients lacking both t(4;14) and del(17p) with a high β2-microglobulin level and with a del(13). The *gray curve* shows patients with either a t(4;14) or a del(17p) in more than 60% of their plasma cells, and a low β2-microglobulin level. Finally, the *pink curve* shows the overall survival of patients with either a t(4;14) or a del(17p) in more than 60% of their plasma cells, and a high β2-microglobulin level. This research was originally published in *Blood*. Ref 18 © the American Society of Hematology

include in their strategy the use of interphase FISH for the assessment of chromosomal changes observed in patients enrolled in those trials. Even though this technique is also technically demanding (plasma cell labeling or sorting), it is much lighter to set up, especially for multicenter trials. Samples can be sent to a reference lab, enabling a huge improvement in cost-effectiveness, and also high reproducibility. So FISH karyotyping is probably the method of choice for the analysis of chromosomal changes in myeloma. Combining Influence of t(4;14), del(17p), along with beta 2-microglobulin level may provide a superior model to predict overall survival (Fig. 2.3).

2.4 Genomic Abnormalities in MM

Genome-wide CGH and SNP DNA arrays have demonstrated their utility to identify acquired chromosome abnormalities in myeloma cells [1, 2, 62–69]. Despite dramatic differences of resolution between metaphase-based CGH and array CGH (aCGH) or high-density SNP arrays (SNP array), the skyline recurrence plot for copy number abnormalities (CNAs) is similar in different studies [2]. High-resolution molecular karyotyping using whole-genome DNA provided molecular evidence

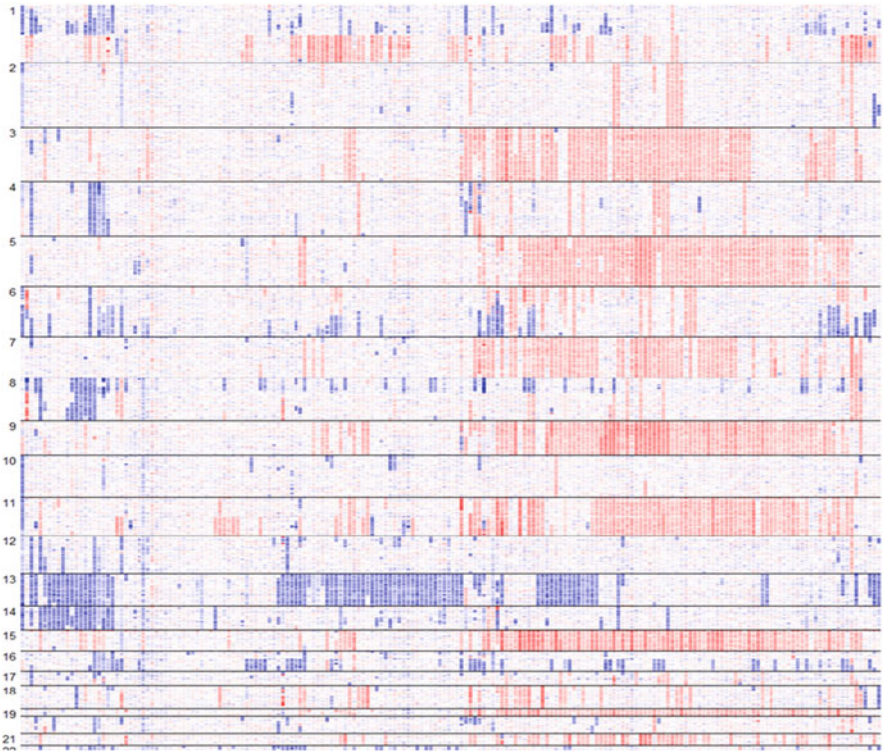


Fig. 2.4 SNP array plot of 192 patients analyzed with the Affymetrix 500 K chip.Legend: each column represents a single patient. SNPs are ordered from chromosome 1p to chromosome 22q. *Blue* represents losses, whereas *red* represents gains. This research was originally published in *Blood*. Ref 18 © the American Society of Hematology

that MM is a heterogeneous genetic disease with an average of at least seven numerical and/or structural chromosomal abnormalities (Fig. 2.4) [1, 2].

MM patients are characterized by highly frequent (>30%) gain of the odd numbered chromosomes 3, 5, 7, 9, 11, 15, 19, and 21, loss of chromosomes 13 and X (in females cases), and gain of 1q and frequent (>10%) deletion of sub-chromosomal material including 1p, 6q, 8p, 12p, 14q, 16p, 16q, and 20p and gain of 6p. Based on these frequent CNAs, MM are clustered into several groups, with one predominant group including the vast majority of hyperdiploid MM (≥47 chromosomes) that almost exclusively harbored chromosome gains (whole or segmental) [2]. Among hyperdiploid MM, a clear survival advantage was demonstrated for either hyperdiploid patients with trisomy 11 [1] or 5q31 gain [2].

Besides their high resolution, genome-wide SNP arrays combine intensity and genotype data to simultaneously evaluate copy number and loss of heterozygosity in cancer cells. The use of adapted analysis tools such as CHAS (http://www.affymetrix.com), CNAG and AsCNAR (http://www.genome.umin.jp), dChip (http://www.dChip.org), and Partek GS (http://www.partek.com) allows to establish both allele frequency and determine allele-specific copy number, facilitating exploration

of acquired uniparental disomy (UPD) and minor populations in cancer. Few studies have analyzed distribution, size, and frequency of UPD in MM [2, 67, 69]. Two mechanisms of acquired UPD can occur in MM: whole-chromosome UPD that arise from a chromosomal segregation error in mitosis and segmental UPD that occur through mitotic recombination events. Whole-chromosome UPD is a rare event, apparently not random, affecting predominantly even chromosomes. Segmental UPD is more frequent and affect preferentially chromosomes 16q and Xq. UPD has no prognostic impact in MM.

2.4.1 Minimal Common Regions of Interest with Prognostic Value

Analysis of genome-wide aCGH or SNP arrays in large series of MM allows identification of recurrent minimal common genetic lesions [1, 2, 69]. These genomic analyses confirmed prognostic significance of 1q21 (*CKS1B*), 8q24 (*MYC*), and 17p13 (*TP53*) and revealed novel loci at 1p, 5q, 8q, 12p, 14q, 16q, 20p, 20q, and 22q, which impact on survival. However, prognostic significance of these lesions has to be confirmed in larger genomic studies using standardized genomic analysis tools.

2.4.2 Genes of Interest Residing in MGCL

Integration of recurrent amplifications and deletions with their correlated gene expression changes identified a number of candidate genes associated with poor survival. Among them, genes with "oncogene-like" pattern defined as genes residing in amplified genomic loci showing significant overexpression in amplified genomic locus included *ADAR*, *CKS1B*, *IL6R*, *ILF2*, *MCL1*, *SHC1*, *UBAP2L*, and *UBE2Q1* at 1q21 locus; *MYC*, *FBXO32* at 8q24; and *YWHAB* at 20q13. Genes with "tumor suppressor gene-like" pattern defined as downregulated genes located in deleted genomic regions included *DFFA* at 1p36; *CD27/EVI1* at 12p13; *CYLD* at 16q12; and *TP53* at 17p13.

2.4.3 Multivariate Analysis of Lesions Independent of Risk Stratification

Based on the frequent genomic lesions (>10%) with the most significant prognostic impact identified in newly diagnosed MM enrolled in IFM trials, we built a survival model. Multivariate analysis retained two adverse genetic markers: amp(1q23.3) and del(12p13.31) and one favorable marker: amp(5q31.3) [2]. Further genomic studies by other cooperative groups are required to evaluate the prognostic impact of the model in patients treated with different regimens.

2.5 Conclusion

To conclude, genetic changes represent probably the most important prognostic factor in MM, as previously shown in other hematological malignancies. Several techniques can be used to identify these factors, including FISH, SNP, or CGH array, but also gene expression profiling (described in another chapter). The goal now will be to define what is (are) the best technique(s) to use to detect these prognostic factors.

References

1. Carrasco DR, Tonon G, Huang Y et al (2006) High-resolution genomic profiles define distinct clinico-pathogenetic subgroups of multiple myeloma patients. Cancer Cell 9:313–325
2. Avet-Loiseau H, Li C, Magrangeas F et al (2009) Prognostic significance of copy-number alterations in multiple myeloma. J Clin Oncol 27:4585–4590
3. Dewald GW, Kyle RA, Hicks GA, Greipp PR (1985) The clinical significance of cytogenetic studies in 100 patients with multiple myeloma, plasma cell leukemia or amyloidosis. Blood 66:380–390
4. Weh HJ, Gutensohn K, Selbach J et al (1993) Karyotype in multiple myeloma and plasma cell leukemia. Eur J Cancer 29A:1269–1273
5. Sawyer JR, Waldron JA, Jagannath S, Barlogie B (1995) Cytogenetic findings in 200 patients with multiple myeloma. Cancer Genet Cytogenet 82:41–49
6. Laï JL, Zandecki M, Mary JY et al (1995) Improved cytogenetics in multiple myeloma: a study of 151 patients including 117 patients at diagnosis. Blood 85:2490–2497
7. Calasanz MJ, Cigudosa JC, Odero MD et al (1997) Cytogenetic analysis of 280 patients with multiple myeloma and related disorders: Primary breakpoints and clinical correlations. Genes Chromosomes Cancer 18:84–93
8. Smadja NV, Bastard C, Brigaudeau C, Leroux D, Fruchart C (2001) Hypodiploidy is a major prognostic factor in multiple myeloma. Blood 98:2229–2238
9. Drach J, Schuster J, Nowotny H et al (1995) Multiple myeloma: high incidence of chromosomal aneuploidy as detected by interphase fluorescence in situ hybridization. Cancer Res 55:3854–3859
10. Drach J, Angerler J, Schuster J et al (1995) Interphase fluorescence in situ hybridization identifies chromosomal abnormalities in plasma cells from patients with monoclonal gammopathy of undetermined significance. Blood 86:3915–3921
11. Zandecki M, Laï JL, Genevieve F et al (1997) Several cytogenetic subclones may be identified within plasma cells from patients with monoclonal gammopathy of undetermined significance, both at diagnosis and during the indolent course of this condition. Blood 90:3682–3690
12. Nishida K, Tamura A, Nakazawa N et al (1997) The Ig heavy chain is frequently involved in chromosomal translocations in multiple myeloma and plasma cell leukemia as detected by in situ hybridization. Blood 90:526–534
13. Fonseca R, Debes-Marun CS, Picken EB et al (2003) The recurrent IgH translocations are highly associated with nonhyperdiploid variant multiple myeloma. Blood 102:2562–2567
14. Debes-Marun CS, Dewald GW, Bryant S et al (2003) Chromosome abnormalities clustering and its implications for pathogenesis and prognosis in myeloma. Leukemia 17:427–436
15. Chng WJ, Winkler JM, Greipp PR et al (2006) Ploidy status rarely changes in myeloma patients at disease progression. Leuk Res 30:266–271
16. Wuillème S, Robillard N, Lodé L et al (2005) Ploidy, as detected by fluorescence in situ hybridization, defines different subgroups in multiple myeloma. Leukemia 19:275–278
17. Chng WJ, Van Wier SA, Ahmann GJ et al (2005) A validated FISH trisomy index demonstrates the hyperdiploid and nonhyperdiploid dichotomy in MGUS. Blood 106:2156–2161

18. Avet-Loiseau H, Attal M, Moreau P et al (2007) Genetic abnormalities and survival in multiple myeloma: the experience of the Intergroupe Francophone du Myélome. Blood 109: 3489–3495
19. Avet-Loiseau H, Li JY, Morineau N et al (1999) Monosomy 13 is associated with the transition of monoclonal gammopathy of undetermined significance to multiple myeloma. Blood 94:2583–2589
20. Zojer N, Konigsberg R, Ackermann J et al (2000) Deletion of 13q14 remains an independent adverse prognostic variable in multiple myeloma despite its frequent detection by interphase fluorescence in situ hybridization. Blood 95:1925–1930
21. Desikan R, Barlogie B, Sawyer J et al (2000) Results of high-dose therapy for 1000 patients with multiple myeloma: durable complete remissions and superior survival in the absence of chromosome 13 abnormalities. Blood 95:4008–4010
22. Facon T, Avet-Loiseau H, Guillerm G et al (2001) Chromosome 13 abnormalities identified by FISH analysis and serum beta2-microglobulin produce a powerful myeloma staging system for patients receiving high-dose therapy. Blood 97:1566–1571
23. Fonseca R, Harrington D, Oken MM et al (2002) Biological and prognostic significance of interphase fluorescence in situ hybridization detection of chromosome 13 abnormalities (delta13) in multiple myeloma: An eastern cooperative oncology group study. Cancer Res 62:715–720
24. Zhan F, Tian E, Bumm K et al (2003) Gene expression profiling of human plasma cell differentiation and classification of multiple myeloma based on similarities to distinct stages of late-stage B-cell development. Blood 101:1128–1140
25. Agnelli L, Bicciato S, Fabris S et al (2007) Integrative genomic analysis reveals distinct transcriptional and genetic features associated with chromosome 13 deletion in multiple myeloma. Haematologica 92:56–65
26. Tricot G, Barlogie B, Jagannath S et al (1995) Poor prognosis in multiple myeloma is associated only with partial or complete deletions of chromosome 13 or abnormalities involving 11q and not with other karyotype abnormalities. Blood 86:4250–4256
27. Shaughnessy J, Tian E, Sawyer J et al (2003) Prognostic impact of cytogenetic and interphase fluorescence in situ hybridization-defined chromosome 13 deletion in multiple myeloma: early results of total therapy II. Br J Haematol 120:44–52
28. Chiecchio L, Protheroe RK, Ibrahim AH et al (2006) Deletion of chromosome 13 detected by conventional cytogenetics is a critical prognostic factor in myeloma. Leukemia 20: 1610–1617
29. Laï JL, Michaux L, Dastugue N et al (1998) Cytogenetics in multiple myeloma: a multicenter study of 24 patients with t(11;14)(q13;q32) or its variant. Cancer Genet Cytogenet 104:133–138
30. Fonseca R, Witzig TE, Gertz MA et al (1998) Multiple myeloma and the translocation t(11;14)(q13;q32): a report on 13 cases. Br J Haematol 101:296–301
31. Bergsagel PL, Chesi M, Nardini E et al (1996) Promiscuous translocations into immunoglobulin heavy chain switch regions in multiple myeloma. Proc Natl Acad Sci USA 93:13931–13936
32. Avet-Loiseau H, Facon T, Grosbois B et al (2002) Oncogenesis of multiple myeloma: 14q32 and 13q chromosomal abnormalities are not randomly distributed, but correlate with natural history, immunological features and clinical presentation. Blood 99:2185–2191
33. Fonseca R, Barlogie B, Bataille R et al (2004) Genetics and cytogenetics of multiple myeloma: a workshop report. Cancer Res 64:1546–1558
34. Chesi M, Bergsagel PL, Brents LA et al (1996) Dysregulation of cyclin D1 by translocation into an IgH gamma switch region in two multiple myeloma cell lines. Blood 88:674–681
35. Janssen JWG, Vaandrager JW, Heuser T et al (2000) Concurrent activation of a novel putative transforming gene, myeov, and cyclin D1 in a subset of multiple myeloma cell lines with t(11;14)(q13;q32). Blood 95:2691–2698
36. Fonseca R, Blood EA, Oken MM et al (2002) Myeloma and the t(11;14)(q13;q32): evidence for a biologically defined unique subset of patients. Blood 99:3735–3741
37. Garand R, Avet-Loiseau R, Accard F et al (2003) t(11;14) and t(4;14) translocations correlated with mature lymphoplasmacytoid and immature morphology, respectively, in multiple myeloma. Leukemia 17:2032–2035

38. Robillard N, Avet-Loiseau H, Garand R et al (2003) CD20 is associated with a small mature plasma cell morphology and t(11;14) in multiple myeloma. Blood 102:1070–1071
39. Moreau P, Facon T, Leleu X et al (2002) Recurrent 14q32 translocations determine the prognosis of multiple myeloma especially in patients receiving intensive chemotherapy. Blood 100:1579–1583
40. Gertz MA, Lacy MQ, Dispenzieri A et al (2005) Clinical implications of t(11;14)(q13;q32), t(4;14)(p16.3;q32), and -17p13 in myeloma patients treated with high-dose therapy. Blood 106:2837–2840
41. Chesi M, Nardini E, Brents LA et al (1997) Frequent Translocation t(4;14)(p16.3;q32.3) in multiple myeloma is associated with increased expression and activating mutations of fibroblast growth factor receptor 3. Nat Genet 16:260–264
42. Chesi M, Nardini E, Lim RSC et al (1998) The t(4;14) translocation in myeloma dysregulates both *FGFR3* and a novel gene, *MMSET*, resulting in *IgH/MMSET* hybrid transcripts. Blood 92:3025–3034
43. Stec I, Wright TJ, Van Ommen GJ et al (1998) *WHSC1*, a 90 kb SET domain-containing gene, expressed in early development and homologous to a drosophila dysmorphy gene maps in the Wolf-Hirschorn syndrome critical region and is fused to *IgH* in t(4;14) multiple myeloma. Hum Mol Genet 7:1071–1082
44. Plowright EE, Li Z, Bergsagel PL et al (2000) Ectopic expression of fibroblast growth factor receptor 3 promotes myeloma cell proliferation and prevents apoptosis. Blood 9:992–998
45. Li Z, Zhu YX, Plowright EE et al (2001) The myeloma-associated oncogene fibroblast growth factor receptor 3 is transforming in hematopoietic cells. Blood 97:2413–2419
46. Keats JJ, Reiman T, Maxwell CA et al (2003) In multiple myeloma, t(4;14)(p16;q32) is an adverse prognostic factor irrespective of FGFR3 expression. Blood 101:1520–1529
47. Santra M, Zhan F, Tian E, Barlogie B, Shaughnessy J (2003) A subset of multiple myeloma harboring the t(4;14)(p16;q32) translocation lacks FGFR3 expression but maintains an IGH/MMSET fusion transcript. Blood 101:2374–2376
48. Moreau P, Attal M, Garban F et al (2007) Heterogeneity of t(4;14) in multiple myeloma. Long-term follow-up of 100 cases treated with tandem transplantation in IFM99 trials. Leukemia 21:2020–2024
49. Avet-Loiseau H, Leleu X, Roussel M et al (2010) Bortezomib Plus Dexamethasone Induction Improves Outcome of Patients With t(4;14) Myeloma but Not Outcome of Patients With del(17p). J Clin Oncol 28(30):4630–4634
50. Chesi M, Bergsagel PL, Shonukan OO et al (1998) Frequent dysregulation of the *c-maf* proto-oncogene at 16q23 by translocation to an Ig locus in multiple myeloma. Blood 91:4457–4463
51. Hurt EM, Wiestner A, Rosenwald A et al (2004) Overexpression of *c-maf* is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma. Cancer Cell 5:191–199
52. Hanamura I, Iida S, Akano Y et al (2001) Ectopic expression of *MAFB* gene in human myeloma cells carrying the t(14;20)(q32;q11) chromosomal translocations. Jpn J Cancer Res 92:638–644
53. Drach J, Ackermann J, Fritz E et al (1998) Presence of a *p53* gene deletion in patients with multiple myeloma predicts for short survival after conventional-dose chemotherapy. Blood 92:802–809
54. Chang H, Qi C, Yi QL et al (2005) *p53* gene deletion detected by fluorescence in situ hybridization is an adverse prognostic factor for patients with multiple myeloma following autologous stem cell transplantation. Blood 105:358–360
55. Portier M, Moles JP, Mazars GR et al (1992) *P53* and *RAS* gene mutations in multiple myeloma. Oncogene 7:2539–2543
56. Preudhomme C, Facon T, Zandecki M et al (1992) Rare occurrence of *P53* gene mutations in multiple myeloma. Br J Haematol 81:440–443

57. Chng WJ, Price-Troska T, Gonzalez-Paz N et al (2007) Clinical significance of TP53 mutation in myeloma. Leukemia 21:582–584
58. Lode L, Eveillard M, Trichet V et al (2010) Mutations in TP53 are exclusively associated with del(17p) in multiple myeloma. Haematologica. 95:1973–1976
59. Hanamura I, Stewart JP, Huang Y et al (2006) Frequent gain of chromosome band 1q21 in plasma-cell dyscrasias detected by fluorescence in situ hybridization: incidence increases from MGUS to relapsed myeloma and is related to prognosis and disease progression following tandem stem-cell transplantation. Blood 108:1724–1732
60. Sawyer JR, Tricot G, Lukacs JL et al (2005) Genomic instability in multiple myeloma: evidence for jumping segmental duplications of chromosome arm 1q. Genes Chromosomes Cancer 42:95–106
61. Fonseca R, Van Wier SA, Chng WJ et al (2006) Prognostic value of chromosome 1q21 gain by fluorescent in situ hybridization and increase CKS1B expression in myeloma. Leukemia 20:2034–2040
62. Cigudosa JC, Rao PH, Calasanz MJ et al (1998) Characterization of nonrandom chromosomal gains and losses in multiple myeloma by comparative genomic hybridization. Blood 91:3007–3010
63. Liebisch P, Viardot A, Bassermann N et al (2003) Value of comparative genomic hybridization and fluorescence in situ hybridization for molecular diagnostics in multiple myeloma. Br J Haematol 122:193–201
64. Gutierrez NC, Garcia JL, Hernandez JM et al (2004) Prognostic and biologic significance of chromosomal imbalances assessed by comparative genomic hybridization in multiple myeloma. Blood 104:2661–2666
65. Keats JJ, Fonseca R, Chesi M et al (2007) Promiscuous mutations activate the noncanonical NF-kappaB pathway in multiple myeloma. Cancer Cell 12:131–144
66. Annunziata CM, Davis RE, Demchenko Y et al (2007) Frequent engagement of the classical and alternative NF-kappaB pathways by diverse genetic abnormalities in multiple myeloma. Cancer Cell 12:115–130
67. Walker BA, Leone PE, Jenner MW et al (2006) Integration of global SNP-based mapping and expression arrays reveals key regions, mechanisms, and genes important in the pathogenesis of multiple myeloma. Blood 108:1733–1743
68. Jenner MW, Leone PE, Walker BA et al (2007) Gene mapping and expression analysis of 16q loss of heterozygosity identifies WWOX and CYLD as being important in clinical outcome in multiple myeloma. Blood 110:3291–3300
69. Walker BA, Leone PE, Chiecchio LA et al (2010) A compendium of myeloma associated chromosomal copy number abnormalities and their prognostic value. Blood 116(15): e56–e65

Chapter 3
Advances in Multiple Myeloma Gene-Expression Profiling

Saad Usmani, B. Barlogie, and J.D. Shaughnessy, Jr.

Abstract Our understanding of myeloma biology has benefited greatly with the advent of new technologies, such as interphase fluorescence in situ hybridization (FISH), spectral karyotyping, comparative genomic hybridization, single-nucleotide polymorphism genotyping, and gene-expression profiling (GEP). Combining these approaches with emerging technologies, such as high-throughput proteomics, microRNA profiling, and whole-genome sequencing, not only broadens the spectrum of molecular variables that can be studied but also poses immense challenges to integrate the complexities of these high-dimensional datasets to improve therapy and management of multiple myeloma (MM). The present chapter focuses on the use of GEP of primary disease to classify the disease, define risk, and elucidate underlying mechanisms that are beginning to change clinical decision-making and inform drug design.

3.1 Historical Perspective

It is very likely that each of the six hallmarks of cancer, outlined in the Hanahan–Weinberg model [1], ultimately causes or is related to reproducible changes in the expression of subsets of genes within clonal cancer cells and that these patterns are

S. Usmani • B. Barlogie
Donna D and Donald M Lambert Laboratory for Myeloma Genetics,
Myeloma Institute for Research and Therapy, University of Arkansas for Medical Sciences,
4301 West Markham, Slot 776, Little, AR, USA

J.D. Shaughnessy, Jr. (✉)
Donna D and Donald M Lambert Laboratory for Myeloma Genetics,
Myeloma Institute for Research and Therapy, University of Arkansas for Medical Sciences,
4301 West Markham, Slot 776, Little Rock, AR, USA

Department of Biostatistics, University of Arkansas for Medical Sciences,
Little Rock, AR, USA
e-mail: shaughnessyjohn@uams.edu

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_3,

exclusive and specific to each malignancy. This hypothesis was difficult to test, however, until the completion of the human genome project [2, 3] and the development of high-throughput tools capable of analyzing the activities of all genes simultaneously [4]. It is now believed that the human genome consists of ~25,000 mRNA-encoding genes, and this complexity is increased by posttranscriptional modifications, such as alternative splicing.

In the mid-1990s, Brown and colleagues revolutionized molecular biology by developing a system that used DNA microarrays to monitor the expression levels of thousands of genes in parallel [4–6]. Cloned DNA fragments immobilized on a solid matrix were used simultaneously to probe mRNA pools from a control source and from the tissue of interest, each labeled with a different fluorescent dye. Building on this concept, more advanced and sensitive high-density oligonucleotide microarrays were developed using photolithography and solid-phase chemistry. These whole-genome high-density oligonucleotide microarrays contain hundreds of thousands of oligonucleotide probes and are now the industry standard [7]. The probes are tightly packed and designed to maximize sensitivity, specificity, and reproducibility, which allows consistent discrimination between specific and background signals and between closely related target sequences [8].

Microarray technology was first used to study cancer in 1996 [9], and De Vos and colleagues were the first to use GEP to study MM in 2001 [10]. In these early experiments, human myeloma cell lines and plasma cell leukemia samples were analyzed on small-scale, filter-based cDNA arrays to identify genes involved in intercellular signaling. In spite of its small scale, this study revealed that key signaling molecules within the Wnt pathway were altered in MM. Subsequently, Stewart et al. used a combination of high-throughput DNA sequencing and microarrays on cells pooled from several cases of plasma cell leukemia to establish a comprehensive list of genes expressed in MM [11]. GEP analysis has evolved into a field of its own and, in many ways, is at the epicenter of translational research in MM.

3.2 Microarray Profiling in MM

3.2.1 MM Cell Procurement Techniques

Because of the heterogeneous nature of MM growth within the bone marrow, with variable percentages of tumor in a given site as low as 5%, molecular profiling of unfractionated bone marrow aspirates complicates interpretation of results. To overcome this limitation, researchers have employed various means of cell enrichment of plasma cells from bone marrow aspirates. Plasma cells typically make up <1% of the cells in healthy human bone marrow, so isolation of sufficient numbers of plasma cells from healthy human marrow made large-scale GEP experiments an impractical endeavor for most laboratories. To isolate sufficient

numbers of cells for GEP, two different but complementary specialized methodologies were developed. Zhan et al. employed automated immunomagnetic bead sorting of plasma cells from large-volume bone marrow aspirates using a monoclonal antibody, BB4, raised against syndecan-1/CD138 [12]; this technique routinely has isolated highly homogeneous populations of healthy plasma cells from both bone marrow and tonsil [13]. To create a source of polyclonal plasma cells from healthy donors, Tarte and colleagues developed a method for in vitro differentiation of peripheral blood B cells [14]. Global GEP of polyclonal plasma cells and healthy bone marrow plasma cells derived from immunomagnetic sorting has revealed not only strong similarities but also distinct and reproducible differences between the two populations and myeloma cells [15, 16], suggesting that polyclonal plasma cells may not fully recapitulate the molecular biology of a bone marrow plasma cell.

3.2.2 Early GEP-Based Studies

Early studies made several contributions to understanding the molecular basis of MM by comparing gene-expression profiles of CD138-enriched plasma cells from the bone marrow of healthy donors and patients with monoclonal gammopathy of undetermined significance (MGUS), newly diagnosed MM, and end-stage MM [12]. These studies uncovered potential clues to the molecular pathogenesis of MM—disease-specific changes in gene expression. Myeloma plasma cells can be clearly distinguished from those of healthy donors based on expression of ~120 of 6,800 genes analyzed. Unsupervised clustering of these early global gene-expression data showed that MM could be divided into four distinct molecular subgroups, MM1–MM4, with MM1 being more like MGUS and MM4 being related to myeloma cell lines. The MM4 group also had a higher incidence of cytogenetic abnormalities (CAs) and high serum levels of beta-2-microglobulin, clinical features historically linked to poor prognosis. Consistent with these data, genes distinguishing MM4 from the other groups were related to cell proliferation. More advanced microarray technologies and larger sample sizes have now further divided MM into seven disease classes (discussed below).

These results provided the first evidence that MM is likely numerous molecular entities that presumably employ different molecular mechanisms to get to a tumor with a common histology, which has enormous clinical implications. First, the high resolution of molecular classifications allows retrospective evaluation of class-specific efficacy of current therapeutic regimens, which is exceedingly important when designing clinical trials. For example, a new drug might not show a significant effect on a given endpoint when considering MM as a whole, but the results might be dramatically different if the endpoint is examined in the context of a particular molecular classification of MM, which might include only 5% of the overall population. Second, identifying the genes whose expression is driving these classes can

inform the use of existing agents that might not have been considered and can direct development of new class-specific drugs.

3.2.3 Early GEP-Based Discoveries of MM Genetic Anomalies

To provide insights into the molecular characterization of plasma cell dyscrasias and to investigate the contributions of specific genetic lesions to the biological and clinical heterogeneity of MM, Mattioli et al. compared the GEP of plasma cells isolated from 7 cases of MGUS, 39 of MM, and 6 of plasma cell leukemia. MM was heterogeneous at the transcriptional level, whereas MGUS was distinguished from plasma cell leukemias and the majority of MM cases by differential expression of genes involved in DNA metabolism and proliferation. The clustering of MM cases was mainly driven by the presence of one of five recurrent translocations involving the immunoglobulin heavy-chain (IGH) locus [16]. For example, overexpression of *CCND2* and genes involved in cell-adhesion pathways was observed in cases with t(14;16) and t(14;20), whereas upregulated genes showed apoptosis-related functions in cases with t(4;14). The peculiar finding in cases with t(11;14) was downregulation of the alpha subunit of the interleukin-6 receptor (IL6R). Finally, cancer-testis antigens were specifically expressed in a subgroup of patients characterized by aggressive clinical evolution of MM [17].

Genomic profiling in a large cohort of primary disease revealed that dysregulated expression of cyclin D might be a universal event in myelomagenesis. Relative to plasma cells from the bone marrow of healthy donors, myeloma plasma cells exhibit increased and/or dysregulated expression of either *CCND1*, *CCND2*, or *CCND3* [18]. *IGH*-mediated translocations can directly activate *CCND1* (11q13) [19] or *CCND3* (6p21) [20]; *MAF*-(16q23)- or *MAFB*-(20q11)-activating translocations lead to their transactivation of adhesion molecules and *CCND2*, which is elevated in t(4;14)-positive tumors [21]. Biallelic dysregulation of *CCND1* occurs in nearly 40% of tumors, most of which are hyperdiploid [18]. Elevated levels of *CCND2* and the absence of IGH translocation spikes characterize a novel form of MM discovered through GEP of primary disease (termed "Low Bone," discussed below) [22]; interestingly, elevated expression of *CCND2* is not an adverse prognostic factor in this setting [23].

3.3 GEP-Based MM Classification

Bergsagel et al. developed a classification schema based on GEP spikes of the five recurrent translocations, specific trisomies, and expression of cyclin D genes in a supervised clustering approach [16]. This led to the identification of about 30 genes, which could segregate MM in to eight translocation/cyclin D (TC) groups. These groups were termed the 11q13/TC1, 6p21/TC2, 4p16/TC3, maf/TC4, D1/TC5,

D1 + D2/TC6, D2/TC7, and none/TC8 classes [18]. The authors proposed that these genetic entities are defined by early, perhaps initiating, oncogenic events. The classes exhibited significant differences in clinical features, such as prevalence of bone disease, frequency distribution at relapse, and progression to extramedullary tumor growth. Agnelli et al. used this class-prediction model on purified plasma cells from 50 MM cases. Although the TC1, TC2, TC4, and TC5 groups were characterized by 112 probe sets, the TC3 samples showed heterogeneous clinical features. The TC2 group, characterized by extra copies of the *CCND1* locus and no IGH translocations or 13q deletion, demonstrated overexpression of genes involved in the regulation of protein translation [24]. The failure to validate all TC classes could be attributed to either the small sample size or the possibility that the TC classification is not robust, and new methods of classification are required when dealing with large datasets.

Conversely, the unsupervised hierarchical clustering allows samples to self-organize based on underlying correlations in gene-expression patterns (Fig. 3.1). Using a training set of 351 MM cases and a test set of nearly 200 newly diagnosed MM cases, Zhan et al. separated MM into seven different reproducible classes (Table 3.1) [22]. These molecular classes correlate with the TC classification but are strongly influenced by distinct gene-expression profiles associated with known genetic lesions, including hyperdiploidy, translocations, cell proliferation, and myeloma cell interactions with the bone marrow microenvironment.

Four (MF, MS, CD-1, and CD-2) of these classes demonstrate an elevated expression of particular genes that result from recurrent chromosomal translocations present in ~40% of MM, which occur due to errors in switch recombination and/or somatic hypermutation [25]. These translocations cause normally silent genes to partner with powerful immunoglobulin enhancer elements, resulting in expression spikes that are detectable in microarray studies. The LB class, characterized by a low incidence of magnetic resonance imaging (MRI)-defined bone lesions, expresses high levels of *CCND2* and a unique constellation of genes, including *endothelin-1*. HY is characterized by low ectopic expression of *CCND1* and overexpression of genes mapping to the odd-numbered chromosomes that typically exhibit trisomy in MM. The PR class is related to high expression levels of proliferation-associated genes rather than a primary genetic lesion. This class likely consists of the other classes, but underlying features are masked by expression of proliferation genes. In the following section, we highlight subsets of 700 differentially expressed genes thought to be significant in disease pathogenesis in different MM molecular classes.

3.3.1 MS Class

This class is characterized by t(4;14)(p16;q32) translocation, which is a high-risk MM entity that predicts poor prognosis [26]. This reciprocal translocation results in hyperactivation of both *FGFR3* and *MMSET/WHSC1* genes [27]. All t(4;14)-positive

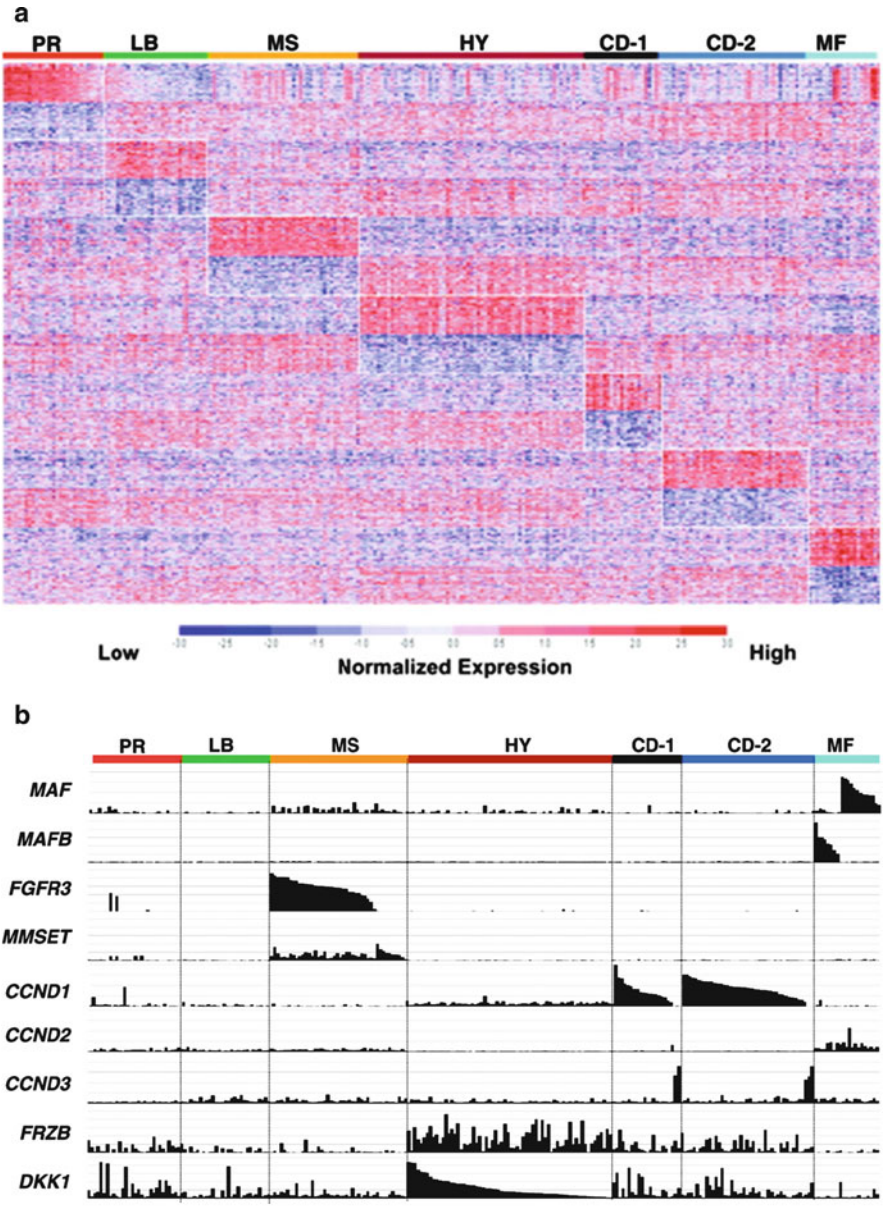


Fig. 3.1 *Classes are characterized by unique GEP patterns*. (*Upper panel*) A supervised clustergram of the expression of 700 genes (50 SAM-defined overexpressed and 50 underexpressed genes from each of the seven classes) across 256 newly diagnosed cases. Genes are indicated along the vertical axis and samples on the horizontal axis. The normalized expression value for each gene is indicated by a *color*, with *red* representing high expression and *blue* representing low expression. (*Lower panel*) The Affymetrix gene-expression signal (expression level: vertical axis) for the

disease expresses elevated levels of *MMSET*, but in about 30% of these cases, expression of *FGFR3* is lost [26, 28]. Because loss of *FGFR3* expression is the only obvious GEP difference between these two types of t(4;14)-positive MM, it appears that *MMSET* plays a central role in driving downstream transcriptional events in the MS class. Furthermore, 25% of MM cases in other classes also exhibit upregulation of *MMSET*, supporting its importance in MM pathogenesis [29]. In a comparison of cases with and without t(4;14), GEP studies identified 127 genes as differentially expressed, including *MMSET* and *CCND2*. Notable genes overexpressed in the MS class, relative to other classes, encode N-cadherin/CDH2, cadherin family member desmoglein2/DSG2, Wnt receptors FZ2 and FZD8, and B-cell oncogene PBX1. Underexpressed genes with potential relevance encode adhesion molecules ICAM4, cadherin 7/CDH7, and transcription factor PAX5.

3.3.2 MF Class

Accounting for ~6% of cases, the MF class of MM is characterized by the t(14;16)(q32;q23) and t(14;20)(q32;q11) translocations, which result in the activation of *c-MAF* and *MAFB* proto-oncogenes, respectively. Cases lacking characteristic *c-MAF* or *MAFB* spikes can be classified as MF, suggesting that other genes of the MAF family may be activated in these cases. Although translocations involving *c-MAF* are seen in <5% of MM cases, *c-MAF* expression is elevated in myeloma cell lines lacking the translocations and in up to 50% of primary samples [21]. These data strongly suggest that *c-MAF* expression may be activated by other mechanisms and attest to the importance of this family of transcription factors in MM pathogenesis. The NF-kB gene-expression signature in the MF class is significantly higher than that in the other classes, with the exception of the LB class (see below) [30]. Clinically, the MF class has relatively low incidence of bone lesions and, consistent with this, has low expression of *DKK1*, a Wnt antagonist produced by myeloma cells and associated with bone disease [31]. In GEP studies aimed at identifying MAFB targets in MM, 284 transcripts were modulated—14 were common to c-MAF and some had functional relationships with MAFB [32]. Additional genes uniquely overexpressed in the MF class that represent known and putative targets of these transcription factors include *NUAK1/ARK5* [33], *NTRK2*, *ARID5A*, *SMARCA1*, *TLR4*, *SPP1*, and *G6MB6*. *CX3CR1* and *ITGB7* are

Fig. 3.1 (continued) mRNA of *MAF*, *MAFB*, *FGFR3*, *MMSET*, *CCND1*, *CCND2*, *CCND3*, *FRZB*, and *DKK1*, within classes presented in the *upper panel*, is indicated. The normalized expression level for each gene across the samples is given by the height of each bar. Note that spiked expression of *CCND1*, *MAF and MAFB*, and *FGFR3 and MMSET* is strongly correlated with specific subgroup designations. Also note that cases retaining the *MMSET* spike but lacking *FGFR3* spikes maintain similar cluster designation, and *MAF* and *MAFB* spikes cluster in the same subgroups. Several *MMSET* and *CCND1* spike cases are evident in the PR class. *CCND3* expression is mutually exclusive of *CCND1* expression. While overexpressed in the HY subgroup, *FRZB* and *DKK1* are significantly underexpressed in LB and MF. Figures reproduced with permission from *Blood*

Table 3.1 Characteristics of validated molecular classes as defined by unsupervised hierarchical clustering

Molecular subtype	% of newly diagnosed patients[a]	Genetic characteristics	Characteristic genes elevated in class	Risk	Features
MS (MMSET)	17	t(4;14)	*FGFR3*, *MMSET*, *CCND2*, *IL6R*	High	Overexpression MMSET and FGFR3′ FGFR3 not evident in ~30%; bone disease is rare
MF (MAF/MAFB)	6	t(14;16) or t(14;20)	*MAF or MAFB*, *CCND2*, *IL6R*	High/moderate	Elevated expression of *CCND2*; bone disease rare; low *DKK1*; High NF-kB signature; low TNF-α-induced gene *TNFAIP8*
CD-1 (C*CND1* or C*CND3*)	6	t(11;14) or t(6;14)	*CCND1 or CCND3*	Low	Few cases express *CCND2 in the absence of CCND1 or CCND3; can have high DKK1*
CD-2 (C*CND1* or ***C****CND3*) + CD20	12	t(11;14) or t(6;14)	*CCND1 or CCND3*, *CD20*, *VPREB3*	Low	Few cases express *CCND2 in the absence o CCND1 or CCND3*
HY (Hyperdiploid)	31	Typical trisomies +3, +5, +7, +9, +11, +15, +19	*GNG11*, *DKK1*, *FRZB*	Moderate	Ectopic expression of *CCND1*; del13, and gain of 1q are rare; high expression of interferon-induced genes
LB (Low Bone disease)	12	Typical HY trisomies; exception is frequent del13, gain of 1q, rare gain of 11	*CCND2*, *CST6*, *ARHE*, *IL6R*	Low	Expression of *CCND2*; low-level *DKK1*, *FRZB*, *CCR2*, *HIF1A*, *SMAD1*; low expression of interferon-induced genes
PR (Proliferation)	10	Made up of all subgroups	*CCNB1*, *CCNB2*, *PCNA*, *MKI67*, *TOP2A*, *TYMS*	High	Overexpression of 1q genes; evolves from other groups

[a]Approximately 13% of newly diagnosed cases were not classified. Unclassified samples typically derived from bone marrow aspirates containing low bone marrow plasmacytosis. This so-called MY subgroup exhibits superior survival relative to other groups. CD138 purified cells from these cases have GEP of signatures consistent with contamination of preparations with of the myeloid, T-cell, B-cell, and plasma cell lineages. Group consists of all molecular classes described above as spikes and class-specific GEP features are evident in most

overexpressed in the MF class [34], consistent with the report that *CCND2*, *CX3CR1*, and *ITGB7* are targets of the c-MAF transcription factor [21]. Expression of *CCND2* is elevated in other disease classes but is highest in the MF class.

3.3.3 CD-1 and CD-2 Classes

The t(11;14)(q13;q32) and t(6;14)(p21;q32) translocations, characteristics of the CD-1 and CD-2 classes, directly activate expression of *CCND1* and *CCND3*, respectively. Tumors with *CCND1* and *CCND3* spikes have gene-expression profiles that cluster membership, suggesting that their activation leads to dysregulation of common downstream pathways.

Nevertheless, *CCND1* and *CCND3* spikes are associated with two distinct, nonoverlapping gene-expression signatures that were used to distinguish the CD-1 and CD-2 classes. CD-2 is characterized by elevated expression of *CD20/MS4A1*, *VPREB3*, and *PAX5*—genes expressed in B cells but normally extinguished in terminally differentiated plasma cells. Of note, CD20 mRNA and protein levels are correlated [22], but cells expressing elevated *PAX5* mRNA do not express the protein [35]. Unlike CD-2, CD-1 lacks expression of *CD59* (potent inhibitor of complement membrane-attack complex), Notch-like protein *NOTCH2NL*, and Notch target gene *HES1*. CD-1 is characterized by overexpression of *KLHL4* (a transcription factor), *INHBE*, *FYN* proto-oncogene, *CEBPB*/*NF-IL6*, and *EVER1* and *EVER2*, two cytoplasmic proteins that colocalize with calnexin, an integral membrane protein of the endoplasmic reticulum [22].

3.3.4 HY Class

Hyperdiploid MM is characterized by trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19, and 21. The HY signature is present in nearly 50% of cases and is associated with hyperdiploid karyotypes in more than 90% of these; however, the signature is also observed in cases that are not identified as hyperdiploid by flow cytometry analyses. Such cases may arise through a similar initiating genetic mechanism (trisomies of odd chromosomes) with clonal evolution that results in DNA loss from other chromosomes, resulting in a DNA complement that is essentially diploid. Genes uniquely overexpressed in the HY class encode guanine nucleotide binding protein, gamma 11/GNG11, Trail/TNFSF10, Wnt signaling antagonists FRZB/SFRP3 and DKK1, and MIP1-alpha chemokine receptor CCR5. Overexpression of several interferon-induced genes, including *OAS2*, *IFI27*, and *IFI35*, is also characteristic of this class. Genes significantly underexpressed in the HY class, relative to the other classes, included *CD52* and genes mapping to chromosome 1q—*TAGLN2*, *CKS1B*, and *OPN3*—whose overexpression has been linked to poor survival [36]. These data are also consistent with aCGH studies that defined disease clusters based on copy number variation [37].

These studies revealed that the classical hyperdiploid trisomies defined a specific subset of MM that lacks gains of 1q and deletion of chromosome 13.

3.3.5 LB Class

LB (*l*ow *b*one disease) is a unique MM class characterized by low incidence of MRI-defined focal bone lesions and lacks evidence of translocation spikes or HY gene-expression features. Along with the absence of MRI-defined focal lesions in the LB class, recent studies integrating positron emission tomography (PET) imaging with GEP also revealed that the LB class is uniquely inversely correlated with F18-fluorodeoxyglucose PET-defined focal lesion number and intensity [38]. This class exhibits overexpression of *endothelin 1/EDN1*, a soluble factor secreted by prostate cancers and that causes osteoblastic metastases of prostate cancer. Interestingly, purified EDN1 induces osteoblast differentiation via suppression of Wnt/β-catenin signaling antagonist *DKK1* [39, 40], and the LB class is characterized by significantly lower expression of *DKK1*, suggesting that EDN1 may downregulate *DKK1* in myeloma cells. LB is also associated with high expression levels of *IL6R*. In contrast to LB, the HY class rarely expresses *IL6R* and expresses high levels of *DKK1* and EDN1 decoy receptor *EDNRB*. These data suggest a potential connection between *EDN1* signaling and *DKK1* production and bone disease in MM.

3.3.6 PR Class

The PR (*pr*oliferation) class is another unique MM class that is characterized by overexpression of numerous genes related to cell-cycle progression and cell proliferation, including *CCNB2*, *CCNB1*, *MCM2*, *CDCA2*, *BUB1*, *CDC2*, and *TYMS*, and cancer-testis antigen genes *MAGEA6*, *MAGEA3*, *GAGE1*, and *GAGE4*. Plasma cells from all MM classes have a higher gene-expression-defined proliferation index (PI) than normal plasma cells, but the PI of the PR class is significantly higher than the non-PR classes and comparable to human myeloma cell lines. Metaphase CAs are present in a very high percentage of cases in the PR class. The PR class is associated with poorer survival than other classes. The PR cases with hyperdiploidy are at higher risk than those with the HY signature. Overexpression of proliferation-associated genes in all non-PR classes, the presence of expression spikes in the PR class, and a shift to the PR class upon disease progression suggest that the PR class is driven by a transformation event due to underlying primary genetic lesions.

3.3.7 Classification Outliers

In the study by Zhan et al., about 25% of newly diagnosed cases could not be classified because gene-expression signatures of myeloid/lymphoid-lineage cells and/or polyclonal

plasma cells predominated and, like the PR signature, prevented unsupervised classification. The presence of translocation spikes in these cases supports the idea that this is not a unique class of MM. This contamination signature appears to hold important clinical implications because patients with this signature have lower levels of bone marrow plasmacytosis, lower incidence of CAs, low beta-2-microglobulin and creatinine, and better event-free survival (EFS) and overall survival (OS) than those without the signature.

3.4 MM-Specific Genetic Features and GEP

3.4.1 GEP and Hyperdiploid Disease

GEP has been used to gain a better understanding of hyperdiploid and nonhyperdiploid MM. A combination of FISH and GEP showed that differential expression of 204 genes, involved with oxidative phosphorylation, protein synthesis, and transcription, can distinguish the two types of disease. The majority of upregulated genes in hyperdiploid disease mapped to the hyperdiploid chromosomes, and 29% of genes upregulated in nonhyperdiploid disease mapped to chromosome 16q [41]; these findings were validated in independent datasets. Hyperdiploid MM was further divided into two distinct molecular and transcriptional entities, one characterized by trisomy 11 and another lacking this feature but harboring chromosome 1q gains and chromosome 13 deletion [37, 41].

It was also observed that hyperdiploid MM is primarily defined by a protein synthesis signature driven through a gene-dosage mechanism, which can further subdivide hyperdiploid MM into four independently validated patient clusters. One prominent cluster was characterized by cancer-testis antigen, proliferation-associated genes, and higher median plasma cell labeling index. This patient population experienced much shorter survival times than those in the other three clusters [42]. Genes involved in tumor necrosis factor alpha/TNF-a and NF-kB signaling and anti-apoptosis characterized another cluster, and these patients had better responses to bortezomib than those in other clusters. This hyperdiploid disease cluster is probably the same disease entity as the hyperdiploid disease characterized by gain of 1q, lack of trisomy 11, and deletion of chromosome 13, as well as the LB class of MM. Studies combining GEP with aCGH revealed that the LB disease is primarily composed of this novel type of hyperdiploid disease [43] and is significantly associated with increased NF-κB activation [30].

3.4.2 GEP and Chromosome 13 Deletion

A study reported on the prognostic implications of all individual CAs with reference to GEP, utilizing a cohort of MM patients followed over 9 years. Among all prognostic factors examined prior to treatment, only nonhyperdiploid disease and deletion of chromosome 13 (del13), alone or in combination, were associated with the shortest EFS and OS [44]. A combination of CAs, GEP, and interphase FISH (to identify del13) on 146 patient samples demonstrated that overexpression

of cell-cycle genes distinguished disease with CA from that without CA. This observation was most apparent for cases lacking FISH-defined del13. Del13 by interphase FISH was significantly associated with reduced expression of a subset of genes mapping to chromosome 13, including *RB1*. The authors proposed that haploinsufficiency of genes mapping to chromosome 13, as well as significant upregulation of *IGF-1R* (insulin-like growth factor receptor), may have an amplifying effect on the expression of cell-cycle genes, providing a molecular explanation for the poor outcome of this subset of MM patients.

Recent data indicate that the historic poor prognosis associated with del13 is related to the presence of other molecular features. A study combined FISH-del13 with GEP on highly purified plasma cells from 80 patients newly diagnosed with MM identified 67 differentially expressed genes that were downregulated in del13 MM (44 genes mapped to Chap. 13, 7 genes to Chap. 11, 3 genes to Chap. 19). FISH-defined del13 was associated with upregulation of genes mapping to 1q21–1q42 and downregulation of genes mapping to 19p and most of chromosome 11 [45].

3.4.3 GEP and Gains of Chromosome 1q

Abnormalities of chromosome 1 are among the most frequent chromosomal alterations in MM, found in about 45% cases [46]; the short arm is associated with deletions and the long arm with amplifications [37]. Gain/amplification of 1q21 increases the risk of MM progression, and incidence of the amplification is significantly higher in relapsed than in newly diagnosed MM [46, 47]. GEP studies comparing MM with and without 1q gains [48] identified 61 genes that distinguished the two groups. In cases with 1q gains, 41 of the 43 upregulated genes mapped to 1q12–q44, whereas most of the 18 downregulated genes were localized to chromosomes 13q (7/18) and 11 (6/18). These data point out that cases with 1q amplifications typically also harbor del13 and lack trisomy 11, features consistent with the LB class.

Gains of 1q are associated with upregulation of genes involved in intracellular vesicle-mediated protein transport including *COPA* and *ARF1*, and *RABIF* and *RAB3GAP2*, associated with the Rab GTPases that regulate membrane-vesicle transport. These findings may also partially account for increased expression of genes encoding proteins involved in energy-production pathways. 1q gains also result in downregulation of three genes involved in protein translation (*RPLP2*, *RPL21*, and *FAU*), which is potentially significant because of the role of ER–Golgi transport system in the survival of B-cell malignancies, including MM [49].

Cases with 1q gains also showed significantly altered expression of genes involved in unfolded protein response (UPR), including upregulation of chaperone gene *CLN3*, *UBAP2L* and *UBE2Q1*, proteasome degradation gene *PSMD4*, and *CASP4* gene involved in UPR-induced apoptosis [48]. Because UPR-induced apoptosis can play an important role in malignant cells' sensitivity to certain drugs,

including bortezomib, these studies suggest that a better understanding of UPR may contribute to important new treatment strategies in MM.

3.4.4 GEP and Deletion of 17p13/TP53

Deletion of 17p13 is considered a high-risk feature in MM, which presumably leads to loss of heterozygosity of *TP53* [50], a tumor suppressor gene that transcriptionally regulates cell-cycle progression and cellular responses to DNA damage. Low expression levels of *TP53* are seen in ~10% of newly diagnosed patients and highly correlate with FISH-defined *TP53* deletion as an independent risk factor [51]. Only a few known p53 target genes correlate with *TP53* expression levels in primary myeloma cells. GEP following ectopic expression of *TP53* in four *TP53*-null cell lines identified 85 significantly differentially expressed genes (50 upregulated, 35 downregulated). Using these target genes, unsupervised hierarchical clustering of myeloma-cell samples from 351 newly diagnosed and 90 relapsed patients revealed two major subgroups that strongly correlated with not only *TP53* expression but also OS. These data indicate that loss of *TP53* expression confers high risk and probably results in deregulation of a novel set of p53 target genes specific to MM [51].

3.5 GEP-Based MM Risk Stratification

3.5.1 GEP-Defined High-Risk MM

While most cases of MM initially respond to treatment, a small subset of patients is refractory to therapy from the outset. Eventually, the majority of initial responders will develop resistance over time. Therefore, survival in patients with MM can show considerable variation, and it is difficult to predict outcome based on current laboratory tests. High-risk MM is routinely defined by laboratory parameters alone or in combinations such as the Durie–Salmon staging system [52] and International Staging System (ISS) [53]. The Bartl grading system was developed based on cell morphology [54], and the presence of abnormal metaphase or interphase cytogenetics [55], high plasma cell labeling index [56], and flow cytometric-defined minimal residual disease have also been employed [57]. To determine a better measure of risk stratification, microarray data were correlated with outcome in two independent cohorts. This led to the identification and validation of a high-risk gene-expression signature in ~15% of newly diagnosed MM (Fig. 3.2). The high-risk signature portends poor prognosis and is evident in a subset of all molecular classes. This 70-gene model of high risk is based on expression patterns of 70 genes featuring increased expression of genes from the q arm and reduced expression of genes from the p arm of chromosome 1 [36].

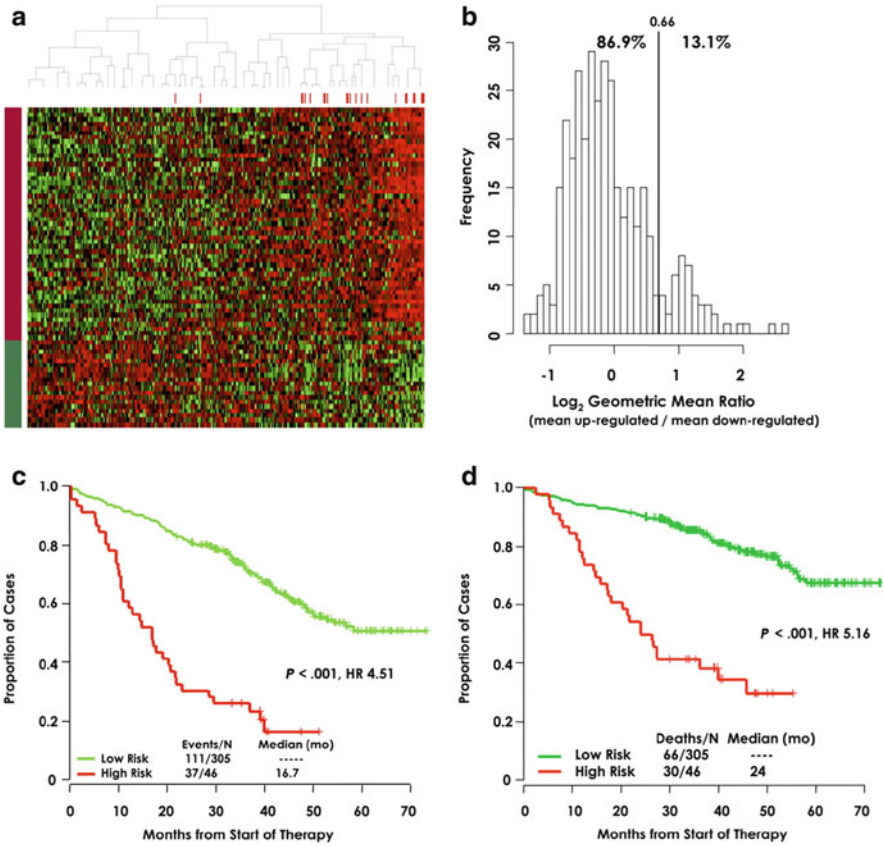


Fig. 3.2 *A GEP-based 70-gene score can define high-risk myeloma* (**a**) Heat map of the 70 genes illustrates remarkably similar expression patterns in CD138^{+} selected tumor cells among 351 newly diagnosed patients. *Red bars above* the patient columns denote patients with disease-related deaths at the time of analysis. The 51 genes in rows designated by the *red bar* on the *left* (*top rows*; upregulated) identified patients in the *upper quartile* of expression at high risk for early disease-related death. The 19 gene rows designated by the *green bar* (downregulated) identified patients in the *lower quartile* of expression at high risk of early disease-related death. (**b**) Frequencies of the risk score defined as the $\log_2$ geometric mean ratio of the 51 quartile-4 genes and 19 quartile-1 genes. This self-normalizing expression ratio has a marked bimodal distribution, consistent with the upper/lower-quartile log-rank differential expression analysis, which was designed to detect genes that define a single high-risk group (13.1%) with an extreme expression distribution. Interpreted as an up/downregulation ratio on the $\log_2$ scale, higher values are associated with poor outcome. The *vertical line* shows the high-risk versus low-risk cutoff for the $\log_2$-scale ratio determined by K-means clustering: the percentage of samples *below* and *above* the cutoff is also shown. Kaplan–Meier estimates of EFS (**c**) and OS (**d**) in low-risk myeloma (*green*) and high-risk myeloma (*red*) showed inferior 5-year actuarial probabilities of EFS (18 vs. 60%, $P < 0.001$; HR = 4.51) and OS (28 vs. 78%, $P < 0.001$; HR = 5.16) in the 13.1% patients with a high-risk signature. Reproduced with permission from *Blood*

Improving on the ISS risk stratification, microarray data were used to develop response and survival classifiers for relapsed disease treated with single-agent bortezomib or high-dose dexamethasone and found to have significant association with outcome [58]. A modified 70/17-gene model was also predictive of poor outcome in relapsed MM [59]. U133A data from newly diagnosed disease validated the 70/17-gene model but also demonstrated that the t(4;14) translocation remained a significant variable for poor outcome [60].

A custom cDNA microarray-based 15-gene model related to MM cell proliferation was also developed to predict high-risk disease and found a hyperdiploid signature being related to better survival [61]. Multivariate analysis comparing the 70/17-gene model with the 15-gene model revealed that the 70/17-gene model was significant in all datasets tested, but the 15-gene model was significant in the bortezomib trials only. These data, together with unpublished studies, suggest that the 70/17-gene model captures more outcome variability than models or indexes of cell proliferation, with the caveat that the R^2 is ~30%.

3.5.2 Progression of Low-Risk to High-Risk at Relapse

GEP on 71 paired newly diagnosed and relapse samples indicated increased 70/17-gene model scores in 80% of cases, and this conversion from low to high risk severely impacted post-relapse survival in 14 of 24 cases (58%) (need to update Fig. 3.3). This quantifiable increase in the high-risk score over time corresponds with increased number of MM cells with gains of chromosome 1q increased over time. This suggests expansion of a dominant MM clone with survival and/or proliferation advantages. The almost universal increase in this risk score during disease evolution suggests that evaluating minimal residual disease may benefit from monitoring this traceable molecular signature, in combination with flow-cytometry-based surrogate. An urgent task is to determine whether a specific baseline GEP signature can prospectively identify which low-risk cases will convert to high risk at relapse, as the 1q21 gains appear to be a final common pathway.

3.5.3 GEP and the Centrosome Index

Centrosome amplification has been implicated as the cause of chromosomal instability in a variety of tumors and may be involved in MM, possibly accounting for the aneuploidy observed in MM. Immunofluorescence staining has shown presence of centrosome amplification in 67% of MGUS [62]. To explore these findings in more depth, a GEP-based centrosome index (CI) was created from gene expression of centrosome proteins and found to have high correlation with the immunofluorescence-detected centrosome amplification. High CI (>4) was associated with poor prognostic genetic features and was an independent prognostic

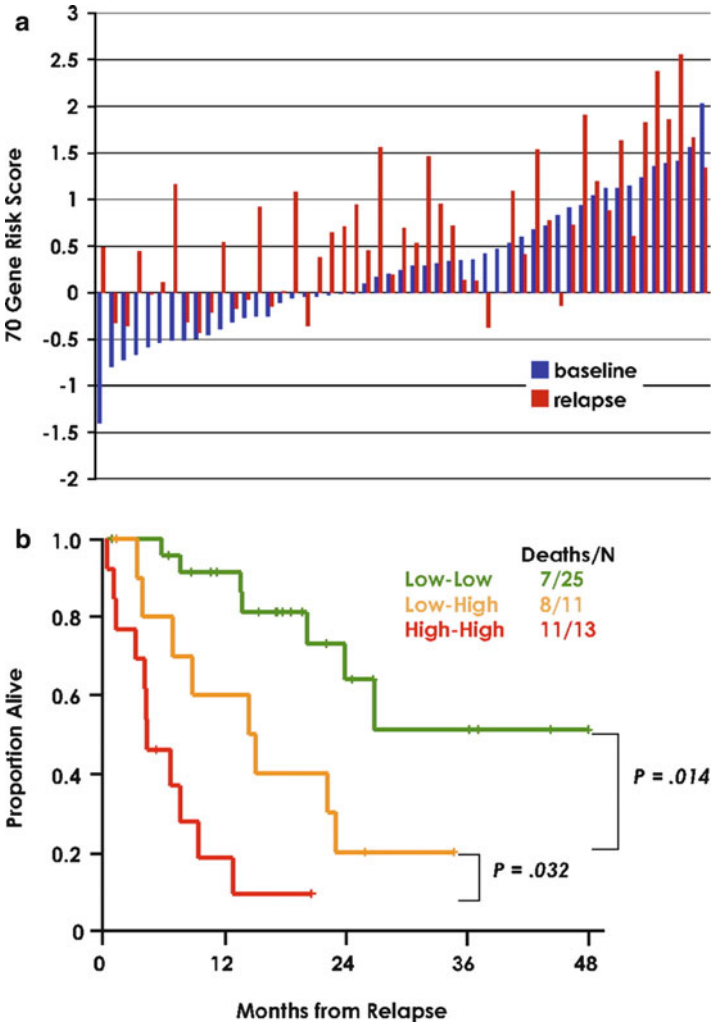


Fig. 3.3 *A 70-gene risk score can increase in relapsed relative to newly diagnosed disease and an increase predicts poor post-relapse survival*. (**a**) The 70-gene risk score in paired diagnostic (*blue*) and relapse (*red*) samples of 51 patients. The gene-expression risk score is indicated to the *left*. Sample pairs are ordered from *left to right* based on lowest baseline score. (**b**) Kaplan–Meier plots of post-relapse survival of the three groups defined by low risk both at diagnosis and relapse (Low–Low), low risk at diagnosis and high risk at relapse (Low–High), and high risk at both time points (High–High). Reproduced with permission from *Blood*

factor in a small cohort of heterogeneously treated cases of MM. Prognostic significance of the CI was subsequently validated in two large cohorts of patients entered into clinical trials, showing that a high CI is a powerful independent prognostic factor in both newly diagnosed and relapsed patients, whether treated by intensive therapy or novel agents [63]. Preclinical studies suggest a potential role of

novel aurora kinase inhibitors in high CI MM, as human myeloma cell lines with higher CIs are more responsive to these compounds (85).

3.5.4 GEP and CD200

Elevated expression of CD200 is an additional prognostic marker that emerged from GEP studies as a high-risk feature in MM [64]. CD200 is a membrane glycoprotein that imparts an immunoregulatory signal through CD200R, leading to suppression of T-cell-mediated immune responses [65]. CD200 expression was predictive for EFS independent of ISS stage or beta-2-microglobin serum levels, but it has not yet been validated in independent datasets or evaluated in the context of OS and all molecular subtypes and models. Because failure of immune surveillance may account for MM progression, CD200 modulation might be an important adjunct to cellular immunotherapy. As a cell-surface protein, CD200 is a potential target for monoclonal antibody therapy, but such strategies will have to consider off-target effects and the critical role of this molecule in immune regulation.

3.6 GEP in MM Therapeutic Decision-Making

3.6.1 GEP and Cancer-Testis Antigen Expression in MM

Cancer-testis antigens are normally expressed in testis and aberrantly expressed in cancer cells but rarely in non-gametogenic tissues, making them attractive targets for cancer immunotherapeutic approaches. GEP studies have discovered that patients newly diagnosed with MM expressed variable numbers of cancer-testis genes (98% expressed at least one, 86% at least two, and 70% at least three) and that expression of six or more cancer-testis genes was associated with shorter EFS [66]. Global GEP studies showed that cancer-testis antigen NY-ESO-1 could be an ideal tumor target antigen for immunotherapy of patients with MM. NY-ESO-1 expression was higher in MM cells from patients with CAs than in those with no CAs, and this observation was more striking in relapsing MM cases [67].

3.6.2 GEP and IGF Signaling

Insulin-like growth factors have been implicated in tumorigenesis and their receptor *IGF-1R* is universally expressed in various hematologic malignancies, including MM, lymphomas, and leukemias. In vitro inhibition of IGF-1R with neutralizing antibody, antagonistic peptide, or selective kinase inhibitor (NVP-ADW742) has

antitumor activity against diverse cell types (particularly MM), including those resistant to conventional cytotoxic agents. Global GEP in MM also delineated modulated intracellular concentrations of key components of the IGF/IGF1-R pathway, including Akt, Raf, and IKK. NVP-ADW742, alone or in combination with cytotoxic chemotherapy, had significant antitumor activity in an orthotopic xenograft MM model, providing in vivo proof-of-principle for therapeutic use of selective IGF-1R inhibitors [68]. Sprynski and colleagues showed that an IGF-1 autocrine loop promoted survival in CD45-negative MM cell lines, while CD45-positive cells required addition of either IL-6 or IGF-1 [69]. GEP analysis in primary disease revealed that elevated expression of IGF-1R and IL6R conferred an adverse prognosis. High expression of both IGF-1R and IL6R is seen most frequently in the MS molecular class, but elevated IGF-1R expression is also seen in other classes and is associated with a poor prognosis. Combining IGF-1-targeted therapy with other novel anti-MM agents may be a useful strategy in the subset of patients with IGF-1R expressing MM.

3.6.3 Pharmacogenomic Studies of Short-Term In Vivo Exposure

Mechanisms underlying chemotherapy resistance are poorly understood, and therapeutic efficacy typically relies on clinical outcome. GEP studies can potentially identify new strategies for avoiding or overcoming drug resistance. Marton et al. [70] and Gray et al. [71] were the first to use microarrays to study the effects of therapeutic agents in yeast, and Cheok et al. were the first to reveal gene-expression patterns in drug responses of human malignancies [72].

Comparative GEP of myeloma cells before and 48 h after single-agent therapy with dexamethasone, thalidomide, or lenalidomide led to the discovery of differentially expressed genes that were prognostic for EFS and OS. Interestingly, these genes were predominantly involved in oxidative stress reactions and actin cytoskeleton rearrangements [73]. Remarkably, gene expression altered by thalidomide in newly diagnosed disease and associated with subsequent survival was also altered by lenalidomide, a thalidomide analogue, and the changes were associated with EFS in a salvage trial of patients with relapsed disease. This finding strongly suggests that these genes are powerful biomarkers, and the similar acute gene-expression responses to two related chemotherapeutic agents may provide important insights into the drugs' potential mechanism(s) of action. These results also highlight the similar acute molecular responses to chemotherapies in both primary and refractory diseases.

GEP studies following therapy with proteasome inhibitor bortezomib in 142 newly diagnosed symptomatic MM cases identified 113 genes with significantly altered expression—predominately downregulated proteasome genes—seen in tumor cells from 76% of patients [74]. The post-bortezomib gene-expression signature was associated with a 3-year survival estimate of >80%, which dramatically contrasts with median survival of <24 months in those with activated proteasome genes. Multivariate

analysis demonstrated that the post-bortezomib score was an independent predictor of outcome that alone accounted for >50% of outcome variability [74]. These data implied that the activation status of proteasome genes in tumor cells after short-term proteasome inhibition is associated with significant outcome differences in patients with MM receiving combination chemotherapy that includes bortezomib.

3.7 Conclusions

Utilizing high-throughput genomic analyses and data-mining techniques, a complete landscape of MM molecular pathogenesis is emerging, and powerful validated prognostic models have been developed. Unsupervised clustering of GEP data of large patient cohorts also have revealed that MM heterogeneity can be accurately cataloged and disease classes defined. Importantly, the improved survival observed in specific classes through the use of new treatments, such as thalidomide, lenalidomide, and bortezomib, buttresses the concept of personalized treatment approaches.

Large-scale gene-expression data and large cohorts of uniformly treated patients with long follow-up times have provided more precise and independent prognostic models for stratifying patients with MM. Investigating GEP changes between baseline and relapse has shed light on the mechanisms underlying MM progression and the nearly universal development of multidrug-resistant MM. Pharmacogenomics studies comparing gene-expression profiles at diagnosis and following short-term single-agent therapy have identified genes associated with drug responses, contributing to mechanistic understanding. This has been critical for improving existing therapies with personalized treatments and led to an explosion in the discovery of new anti-MM therapeutics.

It is well known that MM growth and survival are highly dependent on interactions with the bone marrow microenvironment, and GEP has uncovered many molecular details of these interactions, which might prove to be the Achilles heels of MM. A prominent example was the use of GEP and MRI imaging of bone to learn that myeloma cells aberrantly synthesize DKK1, a potent inhibitor of Wnt/β-catenin signaling, which is required for osteoblast differentiation and function. GEP of whole-bone biopsies that contain tumor cells and all the bone marrow niche cells are also beginning to reveal details of MM pathogenesis and shedding light on potential therapeutic targets.

In summary, GEP is emerging from the research laboratory as a clinical tool with the potential for transforming routine management of MM to truly personalized care. While the majority of MM patients can anticipate long-term disease control via a variety of treatment approaches, patients with molecularly defined high-risk disease do not benefit from current approaches. To address this, clinical trials designed to reduce toxicities in low-risk disease and to test new treatment strategies in high-risk disease are underway. When routinely available, molecular-based classification and risk stratification will meet their potential to shift strategies for MM and possibly cure.

Acknowledgments This work was supported by the National Cancer Institute (grants CA55819-09 and CA97513-01), the Lebow Fund to Cure Myeloma, and the Nancy and Stephen Grand Fund. The manuscript was edited by the Office of Grants and Scientific Publications, University of Arkansas for Medical Sciences.

References

1. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100(1):57–70
2. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG et al (2001) The sequence of the human genome. Science 291(5507):1304–1351
3. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J et al (2001) Initial sequencing and analysis of the human genome. Nature 409(6822):860–921
4. Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270(5235):467–470
5. Shalon D, Smith SJ, Brown PO (1996) A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. Genome Res 6(7):639–645
6. Schena M, Shalon D, Heller R, Chai A, Brown PO, Davis RW (1996) Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. Proc Natl Acad Sci USA 93(20):10614–10619
7. Fodor SP, Read JL, Pirrung MC, Stryer L, Lu AT, Solas D (1991) Light-directed, spatially addressable parallel chemical synthesis. Science 251(4995):767–773
8. Lipshutz RJ, Fodor SP, Gingeras TR, Lockhart DJ (1999) High density synthetic oligonucleotide arrays. Nat Genet 21(1 Suppl):20–24
9. DeRisi J, Penland L, Brown PO, Bittner ML, Meltzer PS, Ray M et al (1996) Use of a cDNA microarray to analyse gene expression patterns in human cancer. Nat Genet 14(4):457–460
10. De Vos J, Couderc G, Tarte K, Jourdan M, Requirand G, Delteil MC et al (2001) Identifying intercellular signaling genes expressed in malignant plasma cells by using complementary DNA arrays. Blood 98(3):771–780
11. Claudio JO, Masih-Khan E, Tang H, Goncalves J, Voralia M, Li ZH et al (2002) A molecular compendium of genes expressed in multiple myeloma. Blood 100(6):2175–2186
12. Zhan F, Hardin J, Kordsmeier B, Bumm K, Zheng M, Tian E et al (2002) Global gene expression profiling of multiple myeloma, monoclonal gammopathy of undetermined significance, and normal bone marrow plasma cells. Blood 99(5):1745–1757
13. Zhan F, Tian E, Bumm K, Smith R, Barlogie B, Shaughnessy J Jr (2003) Gene expression profiling of human plasma cell differentiation and classification of multiple myeloma based on similarities to distinct stages of late-stage B-cell development. Blood 101(3): 1128–1140
14. Tarte K, De Vos J, Thykjaer T, Zhan F, Fiol G, Costes V et al (2002) Generation of polyclonal plasmablasts from peripheral blood B cells: a normal counterpart of malignant plasmablasts. Blood 100(4):1113–1122
15. Tarte K, Zhan F, De Vos J, Klein B, Shaughnessy J Jr (2003) Gene expression profiling of plasma cells and plasmablasts: toward a better understanding of the late stages of B-cell differentiation. Blood 102(2):592–600
16. Bergsagel PL, Kuehl WM (2005) Molecular pathogenesis and a consequent classification of multiple myeloma. J Clin Oncol 23(26):6333–6338
17. Mattioli M, Agnelli L, Fabris S, Baldini L, Morabito F, Bicciato S et al (2005) Gene expression profiling of plasma cell dyscrasias reveals molecular patterns associated with distinct IGH translocations in multiple myeloma. Oncogene 24(15):2461–2473
18. Bergsagel PL, Kuehl WM, Zhan F, Sawyer J, Barlogie B, Shaughnessy J Jr (2005) Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. Blood 106(1):296–303

19. Chesi M, Bergsagel PL, Brents LA, Smith CM, Gerhard DS, Kuehl WM (1996) Dysregulation of cyclin D1 by translocation into an IgH gamma switch region in two multiple myeloma cell lines. Blood 88(2):674–681
20. Shaughnessy J Jr, Gabrea A, Qi Y, Brents L, Zhan F, Tian E et al (2001) Cyclin D3 at 6p21 is dysregulated by recurrent chromosomal translocations to immunoglobulin loci in multiple myeloma. Blood 98(1):217–223
21. Hurt EM, Wiestner A, Rosenwald A, Shaffer AL, Campo E, Grogan T et al (2004) Overexpression of c-maf is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma. Cancer Cell 5(2):191–199
22. Zhan F, Huang Y, Colla S, Stewart JP, Hanamura I, Gupta S et al (2006) The molecular classification of multiple myeloma. Blood 108(6):2020–2028
23. Hanamura I, Huang Y, Zhan F, Barlogie B, Shaughnessy J (2006) Prognostic value of cyclin D2 mRNA expression in newly diagnosed multiple myeloma treated with high-dose chemotherapy and tandem autologous stem cell transplantations. Leukemia 20(7):1288–1290
24. Agnelli L, Bicciato S, Mattioli M, Fabris S, Intini D, Verdelli D et al (2005) Molecular classification of multiple myeloma: a distinct transcriptional profile characterizes patients expressing CCND1 and negative for 14q32 translocations. J Clin Oncol 23(29):7296–7306
25. Kuehl WM, Bergsagel PL (2002) Multiple myeloma: evolving genetic events and host interactions. Nat Rev 2(3):175–187
26. Keats JJ, Reiman T, Maxwell CA, Taylor BJ, Larratt LM, Mant MJ et al (2003) In multiple myeloma, t(4;14)(p16;q32) is an adverse prognostic factor irrespective of FGFR3 expression. Blood 101(4):1520–1529
27. Chesi M, Nardini E, Lim RS, Smith KD, Kuehl WM, Bergsagel PL (1998) The t(4;14) translocation in myeloma dysregulates both FGFR3 and a novel gene, MMSET, resulting in IgH/MMSET hybrid transcripts. Blood 92(9):3025–3034
28. Santra M, Zhan F, Tian E, Barlogie B, Shaughnessy J Jr (2003) A subset of multiple myeloma harboring the t(4;14)(p16;q32) translocation lacks FGFR3 expression but maintains an IGH/MMSET fusion transcript. Blood 101(6):2374–2376
29. Dring AM, Davies FE, Fenton JA, Roddam PL, Scott K, Gonzalez D et al (2004) A global expression-based analysis of the consequences of the t(4;14) translocation in myeloma. Clin Cancer Res 10(17):5692–5701
30. Annunziata CM, Davis RE, Demchenko Y, Bellamy W, Gabrea A, Zhan F et al (2007) Frequent engagement of the classical and alternative NF-kappaB pathways by diverse genetic abnormalities in multiple myeloma. Cancer Cell 12(2):115–130
31. Tian E, Zhan F, Walker R, Rasmussen E, Ma Y, Barlogie B et al (2003) The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. N Engl J Med 349(26):2483–2494
32. van Stralen E, van de Wetering M, Agnelli L, Neri A, Clevers HC, Bast BJ (2009) Identification of primary MAFB target genes in multiple myeloma. Exp Hematol 37(1):78–86
33. Suzuki A, Iida S, Kato-Uranishi M, Tajima E, Zhan F, Hanamura I et al (2005) ARK5 is transcriptionally regulated by the Large-MAF family and mediates IGF-1-induced cell invasion in multiple myeloma: ARK5 as a new molecular determinant of malignant multiple myeloma. Oncogene 24(46):6936–6944
34. Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 98(9):5116–5121
35. Lin P, Mahdavy M, Zhan F, Zhang HZ, Katz RL, Shaughnessy JD (2004) Expression of PAX5 in CD20-positive multiple myeloma assessed by immunohistochemistry and oligonucleotide microarray. Mod Pathol 17(10):1217–1222
36. Shaughnessy JD Jr, Zhan F, Burington BE, Huang Y, Colla S, Hanamura I et al (2007) A validated gene expression model of high-risk multiple myeloma is defined by deregulated expression of genes mapping to chromosome 1. Blood 109(6):2276–2284
37. Carrasco DR, Tonon G, Huang Y, Zhang Y, Sinha R, Feng B et al (2006) High-resolution genomic profiles define distinct clinico-pathogenetic subgroups of multiple myeloma patients. Cancer Cell 9(4):313–325

38. Bartel TB, Haessler J, Brown TL, Shaughnessy JD Jr, van Rhee F, Anaissie E et al (2009) F18-fluorodeoxyglucose positron emission tomography in the context of other imaging techniques and prognostic factors in multiple myeloma. Blood 114(10):2068–2076
39. Yin JJ, Mohammad KS, Kakonen SM, Harris S, Wu-Wong JR, Wessale JL et al (2003) A causal role for endothelin-1 in the pathogenesis of osteoblastic bone metastases. Proc Natl Acad Sci USA 100(19):10954–10959
40. Clines GA, Mohammad KS, Bao Y, Stephens OW, Suva LJ, Shaughnessy JD Jr et al (2007) Dickkopf homolog 1 mediates endothelin-1-stimulated new bone formation. Mol Endocrinol 21(2):486–498
41. Agnelli L, Fabris S, Bicciato S, Basso D, Baldini L, Morabito F et al (2007) Upregulation of translational machinery and distinct genetic subgroups characterise hyperdiploidy in multiple myeloma. Br J Haematol 136(4):565–573
42. Chng WJ, Kumar S, Vanwier S, Ahmann G, Price-Troska T, Henderson K et al (2007) Molecular dissection of hyperdiploid multiple myeloma by gene expression profiling. Cancer Res 67(7):2982–2989
43. Zhou Y, Barlogie B, Herman D, Stephens O, Tian E, Williams D et al (2008) Integration of DNA copy number and gene expression alteration reveal novel insights into the molecular pathogenesis and prognosis of multiple myeloma. Blood (ASH Annual Meeting Abstracts) 12(11):250
44. Shaughnessy J, Jacobson J, Sawyer J, McCoy J, Fassas A, Zhan F et al (2003) Continuous absence of metaphase-defined cytogenetic abnormalities, especially of chromosome 13 and hypodiploidy, ensures long-term survival in multiple myeloma treated with Total Therapy I: interpretation in the context of global gene expression. Blood 101(10):3849–3856
45. Agnelli L, Bicciato S, Fabris S, Baldini L, Morabito F, Intini D et al (2007) Integrative genomic analysis reveals distinct transcriptional and genetic features associated with chromosome 13 deletion in multiple myeloma. Haematologica 92(1):56–65
46. Hanamura I, Stewart JP, Huang Y, Zhan F, Santra M, Sawyer JR et al (2006) Frequent gain of chromosome band 1q21 in plasma-cell dyscrasias detected by fluorescence in situ hybridization: incidence increases from MGUS to relapsed myeloma and is related to prognosis and disease progression following tandem stem-cell transplantation. Blood 108(5):1724–1732
47. Rosinol L, Carrio A, Blade J, Queralt R, Aymerich M, Cibeira MT et al (2005) Comparative genomic hybridisation identifies two variants of smoldering multiple myeloma. Br J Haematol 130(5):729–732
48. Carew JS, Nawrocki ST, Krupnik YV, Dunner K Jr, McConkey DJ, Keating MJ et al (2006) Targeting endoplasmic reticulum protein transport: a novel strategy to kill malignant B cells and overcome fludarabine resistance in CLL. Blood 107(1):222–231
49. Fabris S, Ronchetti D, Agnelli L, Baldini L, Morabito F, Bicciato S et al (2007) Transcriptional features of multiple myeloma patients with chromosome 1q gain. Leukemia 21(5):1113–1116
50. Chng WJ, Price-Troska T, Gonzalez-Paz N, Van Wier S, Jacobus S, Blood E et al (2007) Clinical significance of TP53 mutation in myeloma. Leukemia 21(3):582–584
51. Xiong W, Wu X, Starnes S, Johnson SK, Haessler J, Wang S et al (2008) An analysis of the clinical and biological significance of TP53 loss and the identification of potential novel transcriptional targets of TP53 in multiple myeloma. Blood 112(10):4235–4246
52. Salmon SE, Durie BG (1978) Clinical staging and new therapeutic approaches in multiple myeloma. Recent Results Cancer Res 65:12–20
53. Greipp PR, San Miguel J, Durie BG, Crowley JJ, Barlogie B, Blade J et al (2005) International staging system for multiple myeloma. J Clin Oncol 23(15):3412–3420
54. Bartl R (1988) Histologic classification and staging of multiple myeloma. Hematological oncology 6(2):107–113
55. Fonseca R, Barlogie B, Bataille R, Bastard C, Bergsagel PL, Chesi M et al (2004) Genetics and cytogenetics of multiple myeloma: a workshop report. Cancer Res 64(4):1546–1558
56. Greipp PR, Kumar S (2005) Plasma cell labeling index. Methods Mol Med 113:25–35
57. Paiva B, Vidriales MB, Cervero J, Mateo G, Perez JJ, Montalban MA et al (2008) Multiparameter flow cytometric remission is the most relevant prognostic factor for multiple myeloma patients who undergo autologous stem cell transplantation. Blood 112(10):4017–4023

58. Mulligan G, Mitsiades C, Bryant B, Zhan F, Chng WJ, Roels S et al (2007) Gene expression profiling and correlation with outcome in clinical trials of the proteasome inhibitor bortezomib. Blood 109(8):3177–3188
59. Zhan F, Barlogie B, Mulligan G, Shaughnessy JD Jr, Bryant B (2008) High-risk myeloma: a gene expression based risk-stratification model for newly diagnosed multiple myeloma treated with high-dose therapy is predictive of outcome in relapsed disease treated with single-agent bortezomib or high-dose dexamethasone. Blood 111(2):968–969
60. Chng WJ, Kuehl WM, Bergsagel PL, Fonseca R (2008) Translocation t(4;14) retains prognostic significance even in the setting of high-risk molecular signature. Leukemia 22(2):459–461
61. Decaux O, Lode L, Magrangeas F, Charbonnel C, Gouraud W, Jezequel P et al (2008) Prediction of survival in multiple myeloma based on gene expression profiles reveals cell cycle and chromosomal instability signatures in high-risk patients and hyperdiploid signatures in low-risk patients: a study of the Intergroupe Francophone du Myelome. J Clin Oncol 26(29):4798–4805
62. Chng WJ, Ahmann GJ, Henderson K, Santana-Davila R, Greipp PR, Gertz MA et al (2006) Clinical implication of centrosome amplification in plasma cell neoplasm. Blood 107(9):3669–3675
63. Chng WJ, Braggio E, Mulligan G, Bryant B, Remstein E, Valdez R et al (2008) The centrosome index is a powerful prognostic marker in myeloma and identifies a cohort of patients that might benefit from aurora kinase inhibition. Blood 111(3):1603–1609
64. Moreaux J, Hose D, Reme T, Jourdan E, Hundemer M, Legouffe E et al (2006) CD200 is a new prognostic factor in multiple myeloma. Blood 108(13):4194–4197
65. Gorczynski RM, Lee L, Boudakov I (2005) Augmented Induction of CD4+CD25+ Treg using monoclonal antibodies to CD200R. Transplantation 79(9):1180–1183
66. Condomines M, Hose D, Raynaud P, Hundemer M, De Vos J, Baudard M et al (2007) Cancer/testis genes in multiple myeloma: expression patterns and prognosis value determined by microarray analysis. J Immunol 178(5):3307–3315
67. van Rhee F, Szmania SM, Zhan F, Gupta SK, Pomtree M, Lin P et al (2005) NY-ESO-1 is highly expressed in poor-prognosis multiple myeloma and induces spontaneous humoral and cellular immune responses. Blood 105(10):3939–3944
68. Mitsiades CS, Mitsiades NS, McMullan CJ, Poulaki V, Shringarpure R, Akiyama M et al (2004) Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. Cancer Cell 5(3):221–230
69. Sprynski AC, Hose D, Caillot L, Reme T, Shaughnessy J, Barlogie B et al (2009) The role of IGF-1 as a major growth factor for myeloma cell lines and the prognostic relevance of the expression of its receptor. Blood 113(19):4614–4626
70. Marton MJ, DeRisi JL, Bennett HA, Iyer VR, Meyer MR, Roberts CJ et al (1998) Drug target validation and identification of secondary drug target effects using DNA microarrays. Nat Med 4(11):1293–1301
71. Gray NS, Wodicka L, Thunnissen AM, Norman TC, Kwon S, Espinoza FH et al (1998) Exploiting chemical libraries, structure, and genomics in the search for kinase inhibitors. Science 281(5376):533–538
72. Cheok MH, Yang W, Pui CH, Downing JR, Cheng C, Naeve CW et al (2003) Treatment-specific changes in gene expression discriminate in vivo drug response in human leukemia cells. Nat Genet 34(1):85–90
73. Burington B, Barlogie B, Zhan F, Crowley J, Shaughnessy JD Jr (2008) Tumor cell gene expression changes following short-term in vivo exposure to single agent chemotherapeutics are related to survival in multiple myeloma. Clin Cancer Res 14(15):4821–4829
74. Shaughnessy JD Jr, Qu P, Edmondson P, Herman D, Zhou Y, Tian E et al (2008) Changes in the expression of proteasome genes in tumor cells following short-term proteasome inhibitor therapy predicts survival in multiple myeloma treated with Bortezomib-containing multi-agent chemotherapy. Blood (ASH Annual Meeting Abstracts) 12(11):733

Chapter 4
Growth Factors in Multiple Myeloma

Jérôme Moreaux, Caroline Bret, Karène Mahtouk, Anne-Catherine Sprynski, Dirk Hose, and Bernard Klein

Abstract Numerous studies have been devoted to the identification of myeloma cell growth factors (MGF) and to the signalling pathways leading to survival and/or proliferation of myeloma cells. These MGFs and their receptors may be specific to the myeloma clone or expressed throughout normal plasma cell differentiation. In a first part, we will briefly review the MGFs produced by the bone marrow (BM) microenvironment and then review the major signaling cascades triggered in multiple myeloma cells (MMCs) interacting with their microenvironment.

J. Moreaux
CHU Montpellier, Institute of Research in Biotherapy, Montpellier, France

INSERM, U1040, Montpellier 34197, France

C. Bret • B. Klein (✉)
CHU Montpellier, Institute of Research in Biotherapy, Montpellier, France

INSERM,U1040, Montpellier 34197, France

Université Montpellier 1, Montpellier, France
e-mail: bernard.klein@inserm.fr; b-klein@chu-montpellier.fr

K. Mahtouk • A.-C. Sprynski
INSERM U1040, Montpellier 34197, France

D. Hose
Medizinische Klinik und Poliklinik V, Universitätsklinikum Heidelberg, Heidelberg, Germany

Nationales Centrum für Tumorerkrankungen, Heidelberg, Germany

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_4,

4.1 Myeloma Cell Growth Factors

The biologic behavior of MMCs is not only determined by their genetic background but also by their BM microenvironment. BM environment is a heterogeneous population composed of immune cells, hematopoietic stem cells, BM stromal cells (BMSCs), osteoclasts, osteoblasts, erythrocytes, BM endothelial cells, extracellular matrix proteins, and growth factors. The majority of MGFs is secreted by the BM environment compared to autocrine MGFs [1]. Recent studies have provided a comprehensive overview of MGF expression in the different BM cell subpopulations of MM patients [1–3].

4.1.1 MMC Autocrine Growth Factors

Even if the larger part of MGFs is produced by the BM environment, specific MMC MGFs include two members of the EGF family [neuregulin (NRG) 2 and NRG3], two members of the FGF family (FGF7 and FGF18), and four members of the Wnt family (Wnt4, Wnt10A, Wnt11, and Wnt16). These MGFs are exclusively autocrine for MMCs and only FGF7, Wnt4, and Wnt10A are also expressed in normal plasma cells [1] (Fig. 4.1).

4.1.2 Bone Marrow Stromal Cell-Produced Growth Factors

Adhesion of MMCs to BMSCs activates a multitude of signaling pathways supporting survival and proliferation of MMCs [4]. Within the BM, BMSCs are the main source of MGFs [1]. BMSCs overexpress BDNF, three members of the FGF family (FGF1, FGF2, and FGF5), GDF15, IL-6, JAG1, LIF, VEGF, and two members of the Wnt family (Wnt3 and Wnt5B) [1, 3, 5–7] (Fig. 4.1). The secretion of IL-6 by BMSCs is dependent on MMC/BMSC interaction and VEGF secretion by MMCs [3]. MMC/BMSC interactions are also mediated by integrins, CD40/CD40L, and Notch/Notch ligands [3]. We demonstrated that BMSCs from patients with MM show a specific gene expression profile, with a differential expression of genes coding for proteins involved in MMC growth, angiogenesis, and osteoblast differentiation including IL-6, DKK1, and GDF-15 [5]. Patients' BMSCs significantly overexpressed CD200 [5], an immunomodulator protein associated with a bad prognosis in MM [8].

4.1.3 Osteoclast-Produced Growth Factors

MM patients develop osteolytic bone disease characterized by bone pain, pathologic fractures, and hypercalcemia resulting from the disruption of the coupling

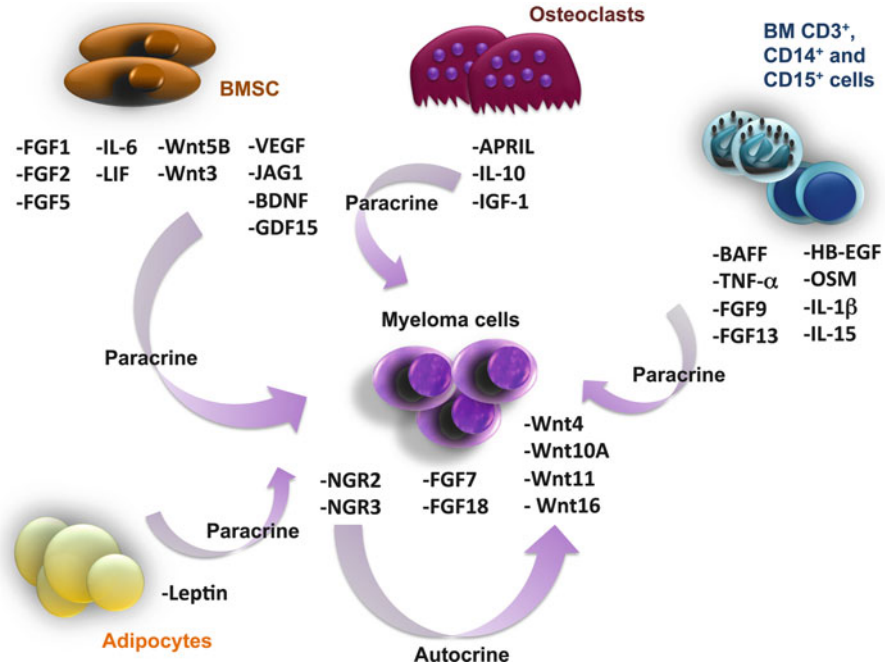


Fig. 4.1 Myeloma cell growth factors produced by the BM microenvironment and/or by myeloma cells themselves

of osteoclastic bone resorption and osteoblastic bone formation [9]. MMCs promote osteoclastic formation directly [10, 11] or indirectly [12, 13] and osteoclasts support MMC survival [2, 11]. Osteoclasts support also the survival of normal plasma cells in vitro [14]. Osteoclasts express specifically four chemokines targeting CCR2 (CCL2, CCL7, CCL8, and CCL13) [2]. CCL7, CCL8, and CCL13 increase significantly the growth of $CCR2^+$ MMCs [2]. Osteoclasts are the main cells in the BM environment that produce various CCR2 chemokines enabling malignant plasma cells attraction and support the growth of MMCs through high expression of IGF-1, APRIL, and IL-10 MGFs [1, 2, 15] (Fig. 4.1).

4.1.4 BM Endothelial Cell-Produced Growth Factors

Angiogenesis is a hallmark of MM progression and disease activity [16]. BM endothelial cells secrete growth factors including VEGF and FGF2. The first use of thalidomide in MM was linked to its anti-angiogenic activity [17]. Thalidomide treatment inhibits secretion of VEGF, FGF2, and HGF MGF [18, 19], and decreased microvessel density within BM was identified only in MM patient responders to thalidomide [20].

4.1.5 BM Immune Cell-Produced Growth Factors

BM polymorphonuclear cells could be an important source of MGFs, especially because they represent around 50% of the cells in the BM. Polymorphonuclear cells highly express BAFF, oncostatin M (OSM), and FGF13 growth factors [1, 15] (Fig. 4.1). Monocytes express high level of BAFF, TNF-α, OSM, IL-1β, IL-15, and HB-EGF [1, 15] (Fig. 4.1).

Dendritic cells (DCs) have been described to be functionally defective in MM patients due to IL-6-, VEGF-, IL-10-, or β2-microglobulin-triggered inhibition of DC maturation [21–24]. Direct MMC/DC interaction increases MM clonogenicity [25]. More recently, a study demonstrated that MMC/DC interaction through CD28 on MMC and CD80/CD86 on DC directly transduces a prosurvival signal to MMC. This interaction induces also the production of IL-6 and the immunosuppressive enzyme IDO by DCs [26].

$CD3^+$ T cells were characterized by a specific overexpression of FGF19 [1]. $CD3^+$ T cells have been identified as the main source of IL-3 in MM patients [27]. In MM, IL-3 inhibited osteoblast formation by an indirect effect mediated by monocytes and macrophages [28]. IL-3 is also involved in osteoclast activation and formation in MM patients [29].

4.1.6 Adipocyte-Produced Growth Factors

Recent data demonstrated that adipocytes should no longer be considered as irrelevant cells in MM biology [30]. Adipocytes could contribute to MMC proliferation, survival, and migration. Adipocytes are the only cells within the MM microenvironment that secreted leptin [30]. Multiplex analyses of growth factors showed that BM adipocytes secrete growth factors involved in MM disease, such as vascular endothelial growth factor, basic fibroblast growth factor (FGF), stem cell factor, VCAM-1, IL-1b, IL-6, IL-10 and IL-12, OSM, and TNF-α [30]. These MGFs are also produced by other BM subpopulations, and the identification of the pivotal cytokines in the interactions between BM adipocytes and MM cells needs further investigations. A study indicated that leptin serum levels were increased in MM patients at diagnosis compared to control patients. Although leptin levels did not increase with advancing MM stages, leptin levels decreased following treatment [31]. Leptin receptor expression on MMCs was described to be predictive for a patients' response to treatment with thalidomide [30].

4.2 Signaling Cascades Connected to MMC Survival and/or Proliferation

Numerous studies have been devoted to the signaling pathways activated by MGFs and leading to survival and/or proliferation of MMCs. MGFs activate one or several major signaling pathways: IL-6, IFNα, IL-10, and IL-21—the JAK/STAT and MAP

kinase pathways; IGF-1, insulin, EGF family, and HGF—the PI-3 kinase/AKT and MAP kinase pathways; BAFF/APRIL and TNF—the NF-kappa B pathway; Jagged 1, Jagged 2, and Delta-like 1 (DLL-1)—the Notch pathway; and Wnt growth factors—the β catenin pathway.

4.2.1 Activation of JAK/STAT and MAP Kinase Pathways: IL-6 Family MGF and IFN-α

IL-6 is a major growth factor for MMCs [32, 33]. BMSCs are the main source of IL-6 [1, 34, 35], and autocrine IL-6 production reflects a highly malignant phenotype [36]. Production of IL-6 by the tumor environment is mainly mediated by IL-1, produced by monocytes and MMCs [1, 33, 37], through PGE2 induction that triggers IL-6 production [37, 38]. IL-6 binds to IL-6 receptor (IL-6R) and the complex IL-6/IL-6R induces the homodimerization of the gp130 IL-6 transducer [39]. Both IL-6R and gp130 are overexpressed in normal plasma cells compared to normal B cells in agreement with the survival effect of IL-6 on plasma cells [40]. Soluble form of IL-6R (sIL-6R) is an agonist, binding IL-6 with a same affinity as membrane IL-6R. IL-6/sIL-6R complex is known to bind and activate gp130 [39]. The major role of IL-6 in the survival and proliferation of MMCs was well established [32, 33]. Anti-IL-6 mAb blocks the survival and proliferation of primary MMCs in vitro [33, 41]. Anti-IL-6 mAb administration inhibited MMC proliferation in patients with terminal disease [42, 43]. High concentration of anti-IL-6 mAb should be injected to block the large IL-6 production in vivo [44]. Elevated serum levels of IL-6 and soluble IL-6R are associated with a poor prognosis in MM patients [45, 46]. MMCs can also directly induce IL-6 production by direct contact with BMSCs [35]. Human myeloma cell lines (HMCLs) whose survival is dependent on the addition of exogenous IL-6 can be obtained from patients with extramedullary proliferation [47] and cover a large part of the molecular heterogeneity of primary MMCs [48]. Transgenic mice with IL-6 gene expression under Eμ promoter develop polyclonal plasmacytosis [49]. Knockdown of IL-6 gene abrogated the generation of malignant plasmacytomas in BALB/C mice primed with mineral oil [50].

An intriguing point is to understand the significance of the low autocrine IL-6 production by MMCs compared to the large amounts secreted by BMSCs. Our group has shown that low autocrine IL-6 production is sufficient to promote cell cycling in HMCLs [51]. At the opposite, survival of HMCLs in vitro necessitates large exogenous IL-6 concentration [51]. We demonstrated that IL-6R expression in MMCs is associated with bad prognosis due to its link with t(4;14) translocation [52].

Other members of the IL-6 family are known to support MMC growth, including OSM, CNTF, IL-11, and LIF [53]. Our recent data demonstrate that these factors are weakly produced by the tumor or its environment suggesting that they are not involved in the emergence of the disease [1]. Interferon-alpha (IFNα) is

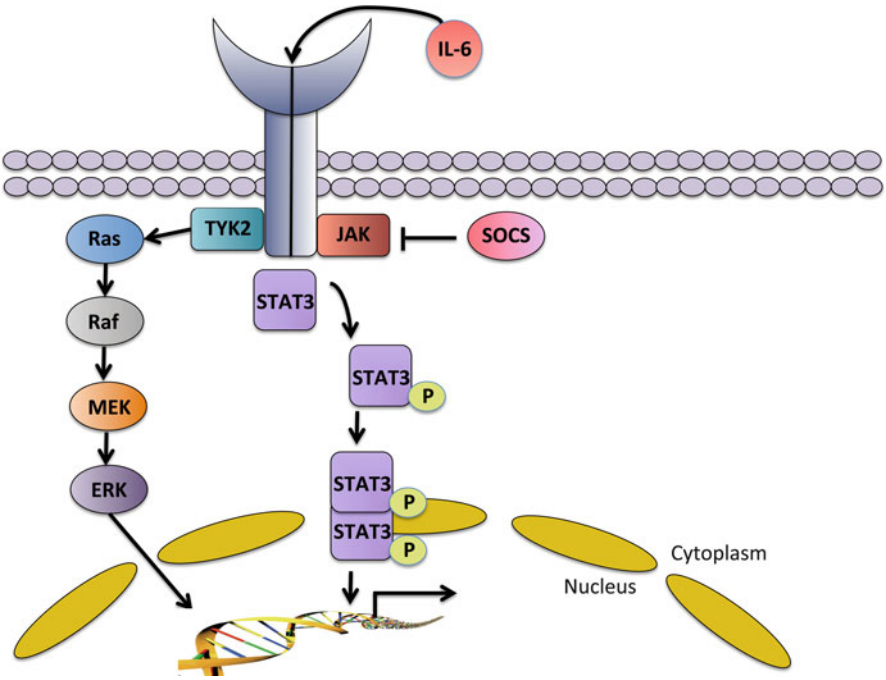


Fig. 4.2 JAK/STAT and MAP kinase signaling pathways

also a MMC survival factor activating the JAK/STAT and MAP kinase pathways [54, 55]. Other groups identified that IFNα could block MMC proliferation probably through the ability of IFNα to induce P19 inhibitor in some HMCL yielding to apoptosis [56]. IL-10 and IL-21 are also identified as MGF [57, 58]. IL-10 is mainly produced by osteoclasts [2] and induces autocrine loops of members of the IL-6 family [59].

These MGFs induce MMC survival through phosphorylation of STAT3 by JAK kinases activated by the gp130 IL-6 transducer or IFN receptor (Fig. 4.2). Blockade of JAK/STAT pathway by AG490 inhibits STAT3 phosphorylation and induces MMC apoptosis [60]. STAT3 could induce the transcription of several anti-apoptotic proteins. Among ten anti-apoptotic and pro-apoptotic proteins, we found that only MCL-1 was regulated by IL-6 or IFNα [61]. Several studies reported that BCL-xL was the main anti-apoptotic protein controlled by IL-6 in MMCs [62, 63]. However, only a blockade of MCL-1, unlike BCL-2 or BCL-xL could inhibit MMC survival [64]. Furthermore, overexpression of MCL-1 is sufficient to promote MMC proliferation independently of IL-6 [65]. We identified a weak AKT phosphorylation in only some IL-6 dependent HMCL [66]. PI-3 kinase/AKT activation can be mediated by STAT3 [67].

4.2.2 Activation of PI-3 and MAP Kinase Pathways: Insulin-Like Growth Factor 1 and Heparin-Binding Growth Factors

4.2.2.1 Insulin-Like Growth Factor 1 (IGF-1)

IGF-1 is an essential growth factor for MMC [52, 68, 69]. Inhibition of IGF-1 pathway reduces MMC growth in vitro and in vivo [70–73]. Osteoclasts are an important source of IGF-1 within the BM [1, 2]. Primary MMCs from patients and HMCLs expressed IGF-1 [1, 2]. IGF-1R is aberrantly expressed by MMCs whereas it is not expressed by normal plasma cells [1]. Furthermore, we have shown that IGF-1R expression is associated with poor prognosis in MM [52]. Plasma IGF-1 level is not significantly increased in MM patients even if it is associated with poor survival [74]. Several IGF-binding proteins, mainly IGF-BP3, circulate at high concentration and neutralize IGF-1 in vivo [75]. Cells may also express IGF-binding protein that contributes to the biological activity of IGF-1 and disrupts the circulating IGF/IGF-BP complexes [1, 76]. MMCs also express the proteoglycan syndecan-1 that can bind these trimeric complexes through IGF-BP3 [1, 76]. Recently, we demonstrated that osteoclasts highly express ADAM28 [77]. ADAM28 displays an enzymatic activity leading to degradation of IGF-BP3 enhancing bioavailability of IGF-1 released from the complex IGF-BP3/IGF-1 [77]. IGF-1 activates mainly PI-3 kinase pathway [52, 78], and its effect is independent of an activation of the JAK/STAT signaling pathway [68, 79]. IGF-1 induces also MAP kinase phosphorylation [52, 78] but MGF activity of IGF-1 was blocked by inhibitor of PI-3K/AKT pathway unlike MAP kinase inhibitor [52, 80]. The PI-3K/AKT pathway in MMCs phosphorylates the P70S6-kinase, forkhead proteins, and the glycogen synthase kinase-3 beta (GSK3b) proteins leading to blockade of apoptosis and activation of cell cycle in various models [80–82] (Fig. 4.3). In MMCs, IGF-1 induces CYCLIN D1 and SKP2 expression and down-regulation of P27/KIP1 [82], and a study reported that PI-3K/AKT pathway may activate expression of several targets of NF-kappa B involved in cell survival including A1/Bfl1, cIAP2, XIAP, survivin, and FLIP [83]. Dominant negative forms of AKT induce inhibition of IL-6-induced proliferation of MMCs, and constitutive activation of AKT enhances tumor growth and protects MMC from DEX-induced apoptosis [84]. A deletion/mutation of PTEN, an inhibitor of the PI-3K/AKT pathway, was reported in some MMCs [85] leading to activation of PI-3K/AKT.

4.2.2.2 Insulin

Insulin, IGF-1, and their receptors are closely related molecules [86]. The role of insulin in MM was poorly studied. Insulin and IGF-1 bind to the receptor of the other one with a weak affinity. Our recent data demonstrated that insulin receptor (INSR) is increased throughout normal plasma cell differentiation [86]. INSR is also expressed by primary MMCs of patients and insulin is an MGF as potent as IGF-1 [86]. Insulin MGF activity requires the presence of insulin/IGF-1 hybrid

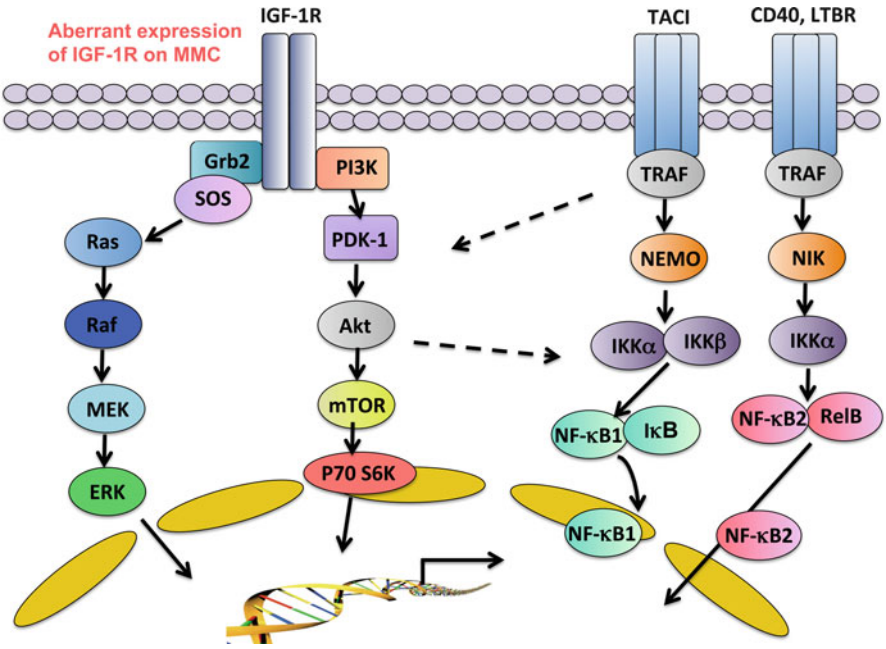


Fig. 4.3 PI-3 kinase, MAP kinase, and NF-κB signaling pathways

receptors, stimulating only INSR$^+$IGF-1R$^+$ MMC and not INSR$^+$IGF-1R$^-$ or INSR$^-$IGF-1R$^-$ MMC [86]. Insulin/IGF-1 hybrid receptors' expression on MMCs was demonstrated by immunoprecipitation, and insulin induced both IGF-1R and INSR phosphorylation suggesting that therapeutic drugs targeting the IGF-1R pathway have to take into account the IGF-1R-mediated insulin MGF activity.

4.2.2.3 Heparin Binding MGF

Syndecan-1 proteoglycan expression is a hallmark of plasma cell differentiation [87, 88]. Syndecan-1 is a proteoglycan concentrating heparin-binding factors on the surface of MMCs, playing likely a major role in MM biology [89, 90]. In patients with MM, all MMCs express syndecan-1 with the exception of preapoptotic cells, which rapidly lose this proteoglycan expression [91]. In addition, among the major cell-surface heparan sulfate proteoglycans, MMCs only express high level of syndecan-1 [90]. Among the heparin-binding growth factors, we will review the MGF activity of epidermal growth factor (EGF) family, hepatocyte growth factor (HGF), and FGF family.

Epidermal Growth Factor Family

We found that MMCs can bind large levels of EGF family molecules through heparan sulfate chain of syndecan-1 molecules [90]. MMCs express the four receptors of EGF family, ErbB1–ErbB4. ErbB1 and ErbB2 are also expressed by normal plasma cells and ErbB3 and ErbB4 are aberrantly expressed by MMCs [92]. A pan-ErbB inhibitor induces strong apoptosis of MMCs cultured for 5 days with their BM environment [93]. EGF members activate PI-3K/AKT and MAPK pathways in MMCs [93]. When the ErbB-specific inhibitor is combined with dexamethasone or anti-IL-6 antibody, apoptosis is significantly increased leading to an almost complete elimination of viable MMCs while non-MMC cells were unaffected [93]. This is likely due to the cooperation between EGF family members and IL-6 to trigger an optimal survival signal to MMC [94]. These data suggest that ErbB inhibitors might improve treatment of patients with MM.

Hepatocyte Growth Factor

HGF/c-MET signaling pathway plays an important role in MM biology [95–98]. Within the BM, HGF is expressed by MMCs, BMSCs, monocytes, and polymorphonuclear cells, and the c-MET receptor is expressed by MMC in 50% of patients [1]. HGF activity is blocked by removal of heparan sulfate chains of syndecan-1 with heparitinase, indicating that syndecan-1 is critical to capture heparin-binding HGF and to present it to its receptor [99]. Serum levels of HGF are significantly increased in MM patients and are associated with a poor prognosis [100]. Low pretreatment HGF concentrations are associated with therapeutic response to bortezomib in patients with MM [101]. Serum concentration of c-MET is not significantly different comparing MM patients and healthy individuals [102]. However, a significant negative correlation between serum c-Met level and disease stage, BM plasma cell percentage, and serum concentration of M-protein was reported. These data suggest a possible role for the c-Met ectodomain as a negative regulator of HGF/c-Met activity [102]. Recently, a study reported a novel mechanism driving the HGF pathway whereby heparanase stimulates an increase in both HGF expression and syndecan-1 shedding to enhance HGF signaling [103]. Our group reported that heparanase expression is a bad prognostic factor in MM [104]. One mechanism is that heparanase can control syndecan-1 gene expression and syndecan-1 shedding into a soluble form that also confers MMC growth advantage [105]. HGF increases bone resorption [106], suggesting that it may also be involved in the abnormal osteoclast resorption in patients with MM.

Fibroblast Growth Factor Family

Analysis of the FGF family was restricted to FGF2 with controversial data concerning the nature of the cells producing FGF2 [34, 107]. Our recent data demonstrated that

BMSCs are the main source of FGF2 [1]. We have shown that other family members are broadly expressed in MM, suggesting that like FGF2, they may contribute to MMC survival and proliferation [1]. The t(4;14) translocation affects the FGF receptor type 3 (FGFR3) in 15% of patients with MM and is associated with a poor prognosis [108, 109]. FGF family members bind syndecan-1 as HB-EGF or HGF, and activation of FGFR3 induces the PI-3K/AKT pathway activation that is critical for MMC survival and proliferation [110]. These data suggest that FGF family could play a role in MM biology.

4.2.3 Activation of NF-Kappa B Pathway: BAFF/APRIL Family Members

APRIL and BAFF are two members of the TNF family that bind to the TNFR-like receptors: transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and B-cell maturation antigen (BCMA). BAFF also binds to BAFF receptor (BAFF-R) [111]. BAFF/APRIL is involved in the survival of normal and malignant B cells and normal plasma cells [111–113]. Activation of BAFF receptor family results in triggering the NF-kappa B pathway and likely other unidentified pathways [113]. Using DNA microarray or FACS analysis, we and others found that MMCs express the two BAFF receptors, BCMA and TACI [66, 114, 115] whereas BAFFR is infrequently expressed by MMCs [114]. In patients with MM, BAFF and APRIL are mainly produced by the BM microenvironment, in particular myeloid cells, monocytes, and osteoclasts [2, 15]. We identified that BAFF and APRIL are potent survival and proliferation factors of MMC, in association with the expression of BCMA or TACI. In addition, BAFF or APRIL can protect MMC from dexamethasone-induced apoptosis [66]. In MMCs, BAFF and APRIL activate MAPK, PI3K/AKT, and NFκB pathways, leading to an upregulation of Mcl-1 and Bcl-2 anti-apoptotic proteins [66, 116]. Of note, several genes coding for NFκB pathway proteins are abnormal in MMCs of 20% of newly diagnosed patients. In particular, an amplification of the *TACI*gene was reported in some MMCs leading to NFκB pathway upregulation [117, 118]. Other gene abnormalities-targeting genes encoding for products amplifying one of the canonical and noncanonical NF-κB pathways (TACI, CD40, LTB-R, NF-κB1, NIK, CYLD, cIAP1-2, TRAF2, TRAF3, NF-κB2) have been described in MM [94, 95, 119, 120] (Fig. 4.3). Our group and others identify syndecan-1 as a coreceptor for APRIL and TACI at the cell surface of MMCs, promoting the activation of an APRIL/TACI pathway that induces survival and proliferation in MMCs [114, 121, 122]. In primary MMCs, the level of TACI expression correlates with a characteristic phenotypic pattern: $TACI^{high}$ MMCs resemble BM mature plasma cells and $TACI^{low}$ MMCs resemble plasmablasts [15]. Same characteristics were observed in HMCLs [123]. The results lead us to perform a phase I trial with a BAFF/APRIL inhibitor, a TACI receptor fused with Fc fragment of human immunoglobulin [124]. TACI-Fc is a dimer. We observed a lack of toxicity of the treatment, and a decrease in total B cell number and also of

polyclonal immunoglobulins indicating an inhibition of the survival of normal plasma cells. A stabilization of the disease was found for some of these patients with refractory disease [124]. Using SCID-hu mice, another group demonstrated that MM patients whose tumors can be selectively classified according to high TACI gene expression may benefit from TACI-Fc treatment [125].

4.2.4 Activation of Notch Pathway: Notch Family

Notch signaling influences multiple processes that govern normal morphogenesis, programmed cell death, and cell proliferation [126]. Notch signaling is implicated in cancer. Overexpression of Notch receptors, ligands, and targets is observed in solid tumors [127–129]. Notch signaling is involved in the pathogenesis of some hematological malignancies [126]. In MM BM, Notch-1, 2, and 3 are expressed by MMCs and BMSCs [1, 130]. Interaction of BMSCs and MMCs induces Notch signaling activation in both MMCs and BMSCs [131, 132], leading to secretion of IL-6, VEGF, and IGF-1 by BMSC [131]. Activation of Notch-1 signaling in MMCs inhibits apoptosis induced by melphalan and mitoxantrone drugs [130]. These data indicate a role of Notch signaling in survival and proliferation of MMCs. Recent data demonstrated that γ-secretase inhibitor induces apoptosis of MMCs via specific inhibition of Notch signaling in vitro and in vivo in mouse models [133]. γ-Secretase inhibitor activity was mediated via Hes-1 and upregulation of Noxa [133]. Inhibition of Notch signaling prevents BM-mediated drug resistance and sensitizes myeloma cells to chemotherapy [133]. Combined inhibition of Notch signaling and Bcl-2/Bcl-xL results in synergistic antimyeloma activity in vitro and in MM mouse models [134]. Notch signaling is also implicated in MMC-induced osteoclast activation [135].

4.2.5 Activation of Wnt Pathway: Wnt Family

The expression of Wnt family members and FRZ receptors has been documented in some HMCLs and primary MMCs, and the biologic relevance of this pathway in the MM has been demonstrated [136, 137]. Our team reported the expression of eight Wnt family members in at least one BM subpopulation in MM patients [1]. Wnt5A is aberrantly overexpressed in MMCs compared to normal plasma cells [1]. Concerning FRZ receptors, FRZ4, FRZ7, and FRZ8 are expressed in more than 90% of the patients. LRP6, which is the required coreceptor of FRZ receptors, is expressed by MMCs [1]. Wnt signaling could be implicated in malignant transformation [138]. Wnt/β-catenin pathway signaling inhibits GSK-3β activity and blocks β-catenin phosphorylation and its degradation by the proteasome leading to β-catenin accumulation in the cytoplasm (Fig. 4.4). Wnt pathway activation by Wnt3A induces accumulation of nuclear β-catenin and increases cell

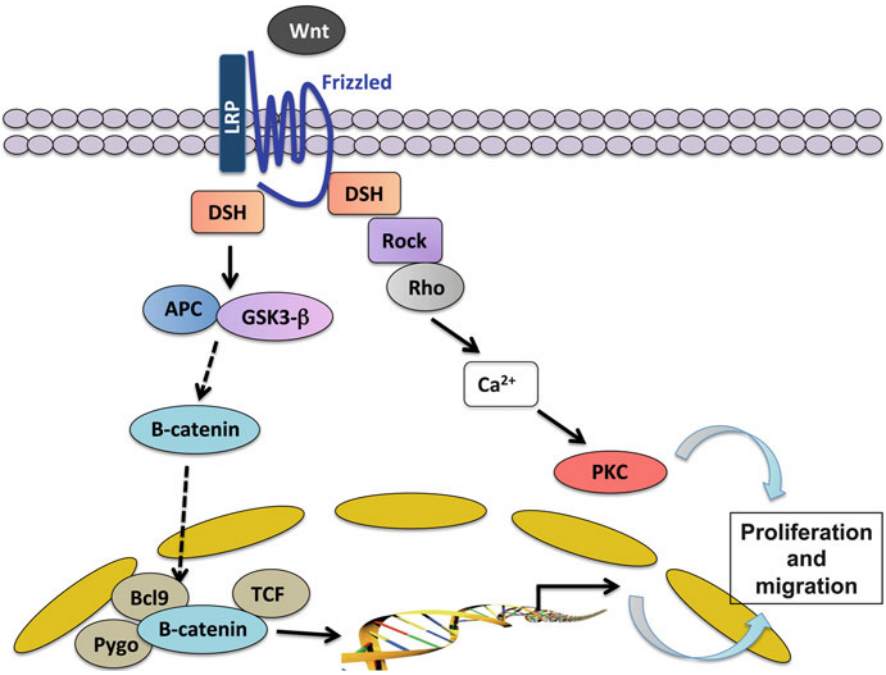


Fig. 4.4 Wnt signaling pathway

proliferation [136]. Wnt pathway activation leads to morphological changes and migration of MMC through alternative Wnt/RhoA signaling and activation of PKC family members [137]. High levels of Dikkopf-1 (DKK-1), an inhibitor of Wnt signaling, in BM plasma are associated with focal bone lesions by inhibiting osteoblasts differentiation [139].

4.3 Hierarchy of MGF in Order to Design Efficient Biologically Based Treatments of MM

Our group and others have reported that IGF-1 is the major growth factor for MMCs [52, 58]. The effect of the larger number of MGFs is dependent, in part, on the activation of IGF-1R by IGF-1. This is the case for IL-6, IL-21, EGF family members, and HGF [52, 58]. IGF-1R is aberrantly expressed by about 50% of primary MMCs of newly diagnosed patients in association with a poor prognosis [52]. This aberrant IGF-1R expression leads to major MMC growth response to IGF-1 and insulin that are abundant in vivo. Anti-IGF-1R monoclonal antibody completely blocked the osteoclast-induced survival of MMCs [2]. IGF-1 activity is linked to the expression of CD45 by MMCs. The phosphatase CD45 can dephosphorylate and inactivate IGF-1R, suggesting that IL-6 is mandatory to support the growth of $CD45^+$ MMCs [71].

IL-6 inhibitors can be useful to induce MMC apoptosis. We have previously found that treatments of patients with terminal disease with anti-IL-6 mAb can block the in vivo proliferation of MMCs and reduce IL-6-related toxicities [42, 43, 140]. A limitation of the anti-IL-6 treatments was the very large production of endogenous IL-6 in patients with MM that could not be neutralized by the anti-IL-6 mAb [44]. IL-6 or others MGFs can increase the resistance of MMCs to MM treatments in vivo. This data indicated that inhibitors of MGFs have to be used in combination with conventional cytotoxic agents. We have documented the rise of large concentrations of IL-6 9 days after high dose melphalan in patients [141]. This large concentration of IL-6 will facilitate melphalan-resistant MMCs to survive within the BM. Our group has performed a phase II trial with anti-IL-6 antibody in association with high dose melphalan [142]. We found that prolonged anti-IL-6 treatment in association with DEX and HDM was feasible and did not affect hematopoietic recovery. It has also shown that patients treated with high-dose melphalan, stem cell transplantation, and anti-IL-6 antibody had a survival advantage when mixed with a large cohort of matched patients treated with melphalan and stem cell transplantation alone [142].

Another major actor in MM biology is syndecan-1. Syndecan-1 is heparan sulfate proteoglycan with covalently attached heparan sulfate chains, which consist of alternating *N*-acetylated glucosamine and D-glucuronic acid units. Heparan sulfate chains with highly modified domains provide specific docking sites for many bioactive molecules, including MGFs, promoting their ability to stimulate MMC growth and survival [90, 114, 143]. Heparan sulfate chain synthesis or syndecan-1 inhibition suppresses the growth of MMCs in vivo [143]. Soluble syndecan-1 produced by MMCs provides an extracellular matrix able to bind circulating IGF-1–IGFBP complexes and to release IGF-1 close to MMCs [52]. Drugs efficiently targeting the heparan sulfate chains of syndecan-1, which is largely expressed by MMCs, will inhibit the biological effect of the majority of MGFs including IGF-1, APRIL, the EGF family members, HGF, and the FGF family.

References

1. Mahtouk K, Moreaux J, Hose D et al (2010) Growth factors in multiple myeloma: a comprehensive analysis of their expression in tumor cells and bone marrow environment using Affymetrix microarrays. BMC Cancer 10:198
2. Moreaux J, Hose D, Kassambara A et al (2011) Osteoclast-gene expression profiling reveals osteoclast-derived CCR2-chemokines promoting myeloma cell migration. Blood 117(4): 1280–1290
3. Podar K, Chauhan D, Anderson KC (2009) Bone marrow microenvironment and the identification of new targets for myeloma therapy. Leukemia 23(1):10–24
4. Hideshima T, Bergsagel PL, Kuehl WM, Anderson KC (2004) Advances in biology of multiple myeloma: clinical applications. Blood 104(3):607–618
5. Corre J, Mahtouk K, Attal M et al (2007) Bone marrow mesenchymal stem cells are abnormal in multiple myeloma. Leukemia 21(5):1079–1088
6. Mitsiades CS, Mitsiades NS, Munshi NC, Richardson PG, Anderson KC (2006) The role of the bone microenvironment in the pathophysiology and therapeutic management of multiple

myeloma: interplay of growth factors, their receptors and stromal interactions. Eur J Cancer 42(11):1564–1573
7. Yasui H, Hideshima T, Richardson PG, Anderson KC (2006) Novel therapeutic strategies targeting growth factor signalling cascades in multiple myeloma. Br J Haematol 132(4):385–397
8. Moreaux J, Hose D, Reme T et al (2006) CD200 is a new prognostic factor in multiple myeloma. Blood 108(13):4194–4197
9. Bataille R, Chappard D, Marcelli C et al (1991) Recruitment of new osteoblasts and osteoclasts is the earliest critical event in the pathogenesis of human multiple myeloma. J Clin Invest 88(1):62–66
10. Han JH, Choi SJ, Kurihara N, Koide M, Oba Y, Roodman GD (2001) Macrophage inflammatory protein-1alpha is an osteoclastogenic factor in myeloma that is independent of receptor activator of nuclear factor kappaB ligand. Blood 97(11):3349–3353
11. Yaccoby S, Wezeman MJ, Henderson A et al (2004) Cancer and the microenvironment: myeloma-osteoclast interactions as a model. Cancer Res 64(6):2016–2023
12. Giuliani N, Colla S, Sala R et al (2002) Human myeloma cells stimulate the receptor activator of nuclear factor-kappa B ligand (RANKL) in T lymphocytes: a potential role in multiple myeloma bone disease. Blood 100(13):4615–4621
13. Pearse RN, Sordillo EM, Yaccoby S et al (2001) Multiple myeloma disrupts the TRANCE/osteoprotegerin cytokine axis to trigger bone destruction and promote tumor progression. Proc Natl Acad Sci USA 98(20):11581–11586
14. Geffroy-Luseau A, Jego G, Bataille R, Campion L, Pellat-Deceunynck C (2008) Osteoclasts support the survival of human plasma cells in vitro. Int Immunol 20(6):775–782
15. Moreaux J, Cremer FW, Reme T et al (2005) The level of TACI gene expression in myeloma cells is associated with a signature of microenvironment dependence versus a plasmablastic signature. Blood 106(3):1021–1030
16. Vacca A, Ribatti D, Presta M et al (1999) Bone marrow neovascularization, plasma cell angiogenic potential, and matrix metalloproteinase-2 secretion parallel progression of human multiple myeloma. Blood 93(9):3064–3073
17. Singhal S, Mehta J, Desikan R et al (1999) Antitumor activity of thalidomide in refractory multiple myeloma. N Engl J Med 341(21):1565–1571
18. Vacca A, Ria R, Semeraro F et al (2003) Endothelial cells in the bone marrow of patients with multiple myeloma. Blood 102(9):3340–3348
19. Vacca A, Scavelli C, Montefusco V et al (2005) Thalidomide downregulates angiogenic genes in bone marrow endothelial cells of patients with active multiple myeloma. J Clin Oncol 23(23):5334–5346
20. Kumar S, Witzig TE, Dispenzieri A et al (2004) Effect of thalidomide therapy on bone marrow angiogenesis in multiple myeloma. Leukemia 18(3):624–627
21. Ratta M, Fagnoni F, Curti A et al (2002) Dendritic cells are functionally defective in multiple myeloma: the role of interleukin-6. Blood 100(1):230–237
22. Brown RD, Pope B, Murray A et al (2001) Dendritic cells from patients with myeloma are numerically normal but functionally defective as they fail to up-regulate CD80 (B7-1) expression after huCD40LT stimulation because of inhibition by transforming growth factor-beta1 and interleukin-10. Blood 98(10):2992–2998
23. Gabrilovich DI, Chen HL, Girgis KR et al (1996) Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. Nat Med 2(10):1096–1103
24. Xie J, Wang Y, Freeman ME 3rd, Barlogie B, Yi Q (2003) Beta 2-microglobulin as a negative regulator of the immune system: high concentrations of the protein inhibit in vitro generation of functional dendritic cells. Blood 101(10):4005–4012
25. Kukreja A, Hutchinson A, Dhodapkar K et al (2006) Enhancement of clonogenicity of human multiple myeloma by dendritic cells. J Exp Med 203(8):1859–1865
26. Nair JR, Carlson LM, Koorella C et al (2011) CD28 expressed on malignant plasma cells induces a prosurvival and immunosuppressive microenvironment. J Immunol 187(3):1243–1253

27. Giuliani N, Morandi F, Tagliaferri S et al (2006) Interleukin-3 (IL-3) is overexpressed by T lymphocytes in multiple myeloma patients. Blood 107(2):841–842
28. Ehrlich LA, Chung HY, Ghobrial I et al (2005) IL-3 is a potential inhibitor of osteoblast differentiation in multiple myeloma. Blood 106(4):1407–1414
29. Lee JW, Chung HY, Ehrlich LA et al (2004) IL-3 expression by myeloma cells increases both osteoclast formation and growth of myeloma cells. Blood 103(6):2308–2315
30. Caers J, Deleu S, Belaid Z et al (2007) Neighboring adipocytes participate in the bone marrow microenvironment of multiple myeloma cells. Leukemia 21(7):1580–1584
31. Alexandrakis MG, Passam FH, Sfiridaki A et al (2004) Serum levels of leptin in multiple myeloma patients and its relation to angiogenic and inflammatory cytokines. Int J Biol Markers 19(1):52–57
32. Kawano M, Hirano T, Matsuda T et al (1988) Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. Nature 332(6159):83–85
33. Klein B, Zhang XG, Jourdan M et al (1989) Paracrine rather than autocrine regulation of myeloma-cell growth and differentiation by interleukin-6. Blood 73(2):517–526
34. Bisping G, Leo R, Wenning D et al (2003) Paracrine interactions of basic fibroblast growth factor and interleukin-6 in multiple myeloma. Blood 101(7):2775–2783
35. Uchiyama H, Barut BA, Mohrbacher AF, Chauhan D, Anderson KC (1993) Adhesion of human myeloma-derived cell lines to bone marrow stromal cells stimulates interleukin-6 secretion. Blood 82(12):3712–3720
36. Frassanito MA, Cusmai A, Iodice G, Dammacco F (2001) Autocrine interleukin-6 production and highly malignant multiple myeloma: relation with resistance to drug-induced apoptosis. Blood 97(2):483–489
37. Costes V, Portier M, Lu ZY, Rossi JF, Bataille R, Klein B (1998) Interleukin-1 in multiple myeloma: producer cells and their role in the control of IL-6 production. Br J Haematol 103(4):1152–1160
38. Hinson RM, Williams JA, Shacter E (1996) Elevated interleukin 6 is induced by prostaglandin E2 in a murine model of inflammation: possible role of cyclooxygenase-2. Proc Natl Acad Sci USA 93(10):4885–4890
39. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F (2003) Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J 374(Pt 1):1–20
40. Cassese G, Arce S, Hauser AE et al (2003) Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. J Immunol 171(4):1684–1690
41. Zhang XG, Bataille R, Widjenes J, Klein B (1992) Interleukin-6 dependence of advanced malignant plasma cell dyscrasias. Cancer 69(6):1373–1376
42. Bataille R, Barlogie B, Lu ZY et al (1995) Biologic effects of anti-interleukin-6 murine monoclonal antibody in advanced multiple myeloma. Blood 86(2):685–691
43. Klein B, Wijdenes J, Zhang XG et al (1991) Murine anti-interleukin-6 monoclonal antibody therapy for a patient with plasma cell leukemia. Blood 78(5):1198–1204
44. Lu ZY, Brailly H, Wijdenes J, Bataille R, Rossi JF, Klein B (1995) Measurement of whole body interleukin-6 (IL-6) production: prediction of the efficacy of anti-IL-6 treatments. Blood 86(8):3123–3131
45. Bataille R, Jourdan M, Zhang XG, Klein B (1989) Serum levels of interleukin 6, a potent myeloma cell growth factor, as a reflect of disease severity in plasma cell dyscrasias. J Clin Invest 84(6):2008–2011
46. Gaillard JP, Bataille R, Brailly H et al (1993) Increased and highly stable levels of functional soluble interleukin-6 receptor in sera of patients with monoclonal gammopathy. Eur J Immunol 23(4):820–824
47. Zhang XG, Gaillard JP, Robillard N et al (1994) Reproducible obtaining of human myeloma cell lines as a model for tumor stem cell study in human multiple myeloma. Blood 83(12):3654–3663
48. Moreaux J, Klein B, Bataille R et al (2011) A high-risk signature for patients with multiple myeloma established from human myeloma cell lines molecular classification. Haematologica 96(4):574–582

49. Suematsu S, Matsuda T, Aozasa K et al (1989) IgG1 plasmacytosis in interleukin 6 transgenic mice. Proc Natl Acad Sci USA 86(19):7547–7551
50. Lattanzio G, Libert C, Aquilina M et al (1997) Defective development of pristane-oil-induced plasmacytomas in interleukin-6-deficient BALB/c mice. Am J Pathol 151(3):689–696
51. Jourdan M, Mahtouk K, Veyrune JL et al (2005) Delineation of the roles of paracrine and autocrine interleukin-6 (IL-6) in myeloma cell lines in survival versus cell cycle. A possible model for the cooperation of myeloma cell growth factors. Eur Cytokine Netw 16(1):57–64
52. Sprynski AC, Hose D, Caillot L et al (2009) The role of IGF-1 as a major growth factor for myeloma cell lines and the prognostic relevance of the expression of its receptor. Blood 113(19):4614–4626
53. Zhang XG, Gu JJ, Lu ZY et al (1994) Ciliary neurotropic factor, interleukin 11, leukemia inhibitory factor, and oncostatin M are growth factors for human myeloma cell lines using the interleukin 6 signal transducer gp130. J Exp Med 179(4):1337–1342
54. Ferlin-Bezombes M, Jourdan M, Liautard J, Brochier J, Rossi JF, Klein B (1998) IFN-alpha is a survival factor for human myeloma cells and reduces dexamethasone-induced apoptosis. J Immunol 161(6):2692–2699
55. Jourdan M, Zhang XG, Portier M, Boiron JM, Bataille R, Klein B (1991) IFN-alpha induces autocrine production of IL-6 in myeloma cell lines. J Immunol 147(12):4402–4407
56. Arora T, Jelinek DF (1998) Differential myeloma cell responsiveness to interferon-alpha correlates with differential induction of p19(INK4d) and cyclin D2 expression. J Biol Chem 273(19):11799–11805
57. Lu ZY, Zhang XG, Rodriguez C et al (1995) Interleukin-10 is a proliferation factor but not a differentiation factor for human myeloma cells. Blood 85(9):2521–2527
58. Menoret E, Maiga S, Descamps G et al (2008) IL-21 stimulates human myeloma cell growth through an autocrine IGF-1 loop. J Immunol 181(10):6837–6842
59. Gu ZJ, Costes V, Lu ZY et al (1996) Interleukin-10 is a growth factor for human myeloma cells by induction of an oncostatin M autocrine loop. Blood 88(10):3972–3986
60. De Vos J, Jourdan M, Tarte K, Jasmin C, Klein B (2000) JAK2 tyrosine kinase inhibitor tyrphostin AG490 downregulates the mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT) pathways and induces apoptosis in myeloma cells. Br J Haematol 109(4):823–828
61. Jourdan M, De Vos J, Mechti N, Klein B (2000) Regulation of Bcl-2-family proteins in myeloma cells by three myeloma survival factors: interleukin-6, interferon-alpha and insulin-like growth factor 1. Cell Death Differ 7(12):1244–1252
62. Catlett-Falcone R, Landowski TH, Oshiro MM et al (1999) Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity 10(1):105–115
63. Puthier D, Derenne S, Barille S et al (1999) Mcl-1 and Bcl-xL are co-regulated by IL-6 in human myeloma cells. Br J Haematol 107(2):392–395
64. Derenne S, Monia B, Dean NM et al (2002) Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. Blood 100(1):194–199
65. Jourdan M, Veyrune JL, Vos JD, Redal N, Couderc G, Klein B (2003) A major role for Mcl-1 antiapoptotic protein in the IL-6-induced survival of human myeloma cells. Oncogene 22(19):2950–2959
66. Moreaux J, Legouffe E, Jourdan E et al (2004) BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone. Blood 103(8): 3148–3157
67. Pfeffer LM, Mullersman JE, Pfeffer SR, Murti A, Shi W, Yang CH (1997) STAT3 as an adapter to couple phosphatidylinositol 3-kinase to the IFNAR1 chain of the type I interferon receptor. Science 276(5317):1418–1420

68. Ferlin M, Noraz N, Hertogh C, Brochier J, Taylor N, Klein B (2000) Insulin-like growth factor induces the survival and proliferation of myeloma cells through an interleukin-6-independent transduction pathway. Br J Haematol 111(2):626–634
69. Georgii-Hemming P, Wiklund HJ, Ljunggren O, Nilsson K (1996) Insulin-like growth factor I is a growth and survival factor in human multiple myeloma cell lines. Blood 88(6):2250–2258
70. Descamps G, Gomez-Bougie P, Venot C, Moreau P, Bataille R, Amiot M (2009) A humanised anti-IGF-1R monoclonal antibody (AVE1642) enhances Bortezomib-induced apoptosis in myeloma cells lacking CD45. Br J Cancer 100(2):366–369
71. Descamps G, Wuilleme-Toumi S, Trichet V et al (2006) CD45neg but Not CD45pos human myeloma cells are sensitive to the inhibition of IGF-1 signaling by a murine anti-IGF-1R monoclonal antibody, mAVE1642. J Immunol 177(6):4218–4223
72. Menu E, Jernberg-Wiklund H, Stromberg T et al (2006) Inhibiting the IGF-1 receptor tyrosine kinase with the cyclolignan PPP: an in vitro and in vivo study in the 5T33MM mouse model. Blood 107(2):655–660
73. Stromberg T, Ekman S, Girnita L et al (2006) IGF-1 receptor tyrosine kinase inhibition by the cyclolignan PPP induces G2/M-phase accumulation and apoptosis in multiple myeloma cells. Blood 107(2):669–678
74. Standal T, Borset M, Lenhoff S et al (2002) Serum insulinlike growth factor is not elevated in patients with multiple myeloma but is still a prognostic factor. Blood 100(12):3925–3929
75. Duan C (2002) Specifying the cellular responses to IGF signals: roles of IGF-binding proteins. J Endocrinol 175(1):41–54
76. Beattie J, Phillips K, Shand JH, Szymanowska M, Flint DJ, Allan GJ (2005) Molecular recognition characteristics in the insulin-like growth factor (IGF)-insulin-like growth factor binding protein-3/5 (IGFBP-3/5) heparin axis. J Mol Endocrinol 34(1):163–175
77. Bret C, Hose D, Reme T et al (2011) Gene expression profile of ADAMs and ADAMTSs metalloproteinases in normal and malignant plasma cells and in the bone marrow environment. Exp Hematol 39(5):546.e8–557.e8
78. Ge NL, Rudikoff S (2000) Insulin-like growth factor I is a dual effector of multiple myeloma cell growth. Blood 96(8):2856–2861
79. Jelinek DF, Witzig TE, Arendt BK (1997) A role for insulin-like growth factor in the regulation of IL-6-responsive human myeloma cell line growth. J Immunol 159(1):487–496
80. Qiang YW, Kopantzev E, Rudikoff S (2002) Insulinlike growth factor-I signaling in multiple myeloma: downstream elements, functional correlates, and pathway cross-talk. Blood 99(11):4138–4146
81. Hideshima T, Nakamura N, Chauhan D, Anderson KC (2001) Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. Oncogene 20(42): 5991–6000
82. Pene F, Claessens YE, Muller O et al (2002) Role of the phosphatidylinositol 3-kinase/Akt and mTOR/P70S6-kinase pathways in the proliferation and apoptosis in multiple myeloma. Oncogene 21(43):6587–6597
83. Mitsiades CS, Mitsiades N, Poulaki V et al (2002) Activation of NF-kappaB and upregulation of intracellular anti- apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. Oncogene 21(37):5673–5683
84. Hsu JH, Shi Y, Hu L, Fisher M, Franke TF, Lichtenstein A (2002) Role of the AKT kinase in expansion of multiple myeloma clones: effects on cytokine-dependent proliferative and survival responses. Oncogene 21(9):1391–1400
85. Ge NL, Rudikoff S (2000) Expression of PTEN in PTEN-deficient multiple myeloma cells abolishes tumor growth in vivo. Oncogene 19(36):4091–4095
86. Sprynski AC, Hose D, Kassambara A et al (2010) Insulin is a potent myeloma cell growth factor through insulin/IGF-1 hybrid receptor activation. Leukemia 24(11):1940–1950

87. Costes V, Magen V, Legouffe E et al (1999) The Mi15 monoclonal antibody (anti-syndecan-1) is a reliable marker for quantifying plasma cells in paraffin-embedded bone marrow biopsy specimens. Hum Pathol 30(12):1405–1411
88. Wijdenes J, Vooijs WC, Clement C et al (1996) A plasmocyte selective monoclonal antibody (B-B4) recognizes syndecan-1. Br J Haematol 94(2):318–323
89. Bret C, Hose D, Reme T et al (2009) Expression of genes encoding for proteins involved in heparan sulphate and chondroitin sulphate chain synthesis and modification in normal and malignant plasma cells. Br J Haematol 145(3):350–368
90. Mahtouk K, Cremer FW, Reme T et al (2006) Heparan sulphate proteoglycans are essential for the myeloma cell growth activity of EGF-family ligands in multiple myeloma. Oncogene 25(54):7180–7191
91. Jourdan M, Ferlin M, Legouffe E et al (1998) The myeloma cell antigen syndecan-1 is lost by apoptotic myeloma cells. Br J Haematol 100(4):637–646
92. Mahtouk K, Hose D, Reme T et al (2005) Expression of EGF-family receptors and amphiregulin in multiple myeloma. Amphiregulin is a growth factor for myeloma cells. Oncogene 24(21):3512–3524
93. Mahtouk K, Jourdan M, De Vos J et al (2004) An inhibitor of the EGF receptor family blocks myeloma cell growth factor activity of HB-EGF and potentiates dexamethasone or anti-IL-6 antibody-induced apoptosis. Blood 103(5):1829–1837
94. Wang YD, De Vos J, Jourdan M et al (2002) Cooperation between heparin-binding EGF-like growth factor and interleukin-6 in promoting the growth of human myeloma cells. Oncogene 21(16):2584–2592
95. Borset M, Seidel C, Hjorth-Hansen H, Waage A, Sundan A (1999) The role of hepatocyte growth factor and its receptor c-Met in multiple myeloma and other blood malignancies. Leuk Lymphoma 32(3–4):249–256
96. Derksen PW, de Gorter DJ, Meijer HP et al (2003) The hepatocyte growth factor/Met pathway controls proliferation and apoptosis in multiple myeloma. Leukemia 17(4):764–774
97. Hov H, Holt RU, Ro TB et al (2004) A selective c-met inhibitor blocks an autocrine hepatocyte growth factor growth loop in ANBL-6 cells and prevents migration and adhesion of myeloma cells. Clin Cancer Res 10(19):6686–6694
98. Du W, Hattori Y, Yamada T et al (2007) NK4, an antagonist of hepatocyte growth factor (HGF), inhibits growth of multiple myeloma cells: molecular targeting of angiogenic growth factor. Blood 109(7):3042–3049
99. Derksen PW, Keehnen RM, Evers LM, van Oers MH, Spaargaren M, Pals ST (2002) Cell surface proteoglycan syndecan-1 mediates hepatocyte growth factor binding and promotes Met signaling in multiple myeloma. Blood 99(4):1405–1410
100. Seidel C, Borset M, Turesson I, Abildgaard N, Sundan A, Waage A (1998) Elevated serum concentrations of hepatocyte growth factor in patients with multiple myeloma. The Nordic Myeloma Study Group. Blood 91(3):806–812
101. Ludek P, Hana S, Zdenek A et al (2010) Treatment response to bortezomib in multiple myeloma correlates with plasma hepatocyte growth factor concentration and bone marrow thrombospondin concentration. Eur J Haematol 84(4):332–336
102. Wader K, Fagerli U, Holt R, Borset M, Sundan A, Waage A (2011) Soluble c-Met in serum of multiple myeloma patients: correlation with clinical parameters. Eur J Haematol 87(5):394–399
103. Ramani VC, Yang Y, Ren Y, Nan L, Sanderson RD (2011) Heparanase plays a dual role in driving hepatocyte growth factor (HGF) signaling by enhancing HGF expression and activity. J Biol Chem 286(8):6490–6499
104. Mahtouk K, Hose D, Raynaud P et al (2007) Heparanase influences expression and shedding of syndecan-1, and its expression by the bone marrow environment is a bad prognostic factor in multiple myeloma. Blood 109(11):4914–4923
105. Yang Y, Macleod V, Miao HQ et al (2007) Heparanase enhances syndecan-1 shedding: a novel mechanism for stimulation of tumor growth and metastasis. J Biol Chem 282(18):13326–13333

106. Hjertner O, Torgersen ML, Seidel C et al (1999) Hepatocyte growth factor (HGF) induces interleukin-11 secretion from osteoblasts: a possible role for HGF in myeloma-associated osteolytic bone disease. Blood 94(11):3883–3888
107. Colla S, Morandi F, Lazzaretti M et al (2003) Do human myeloma cells directly produce basic FGF? Blood 102(8):3071–3072, author reply 3072–3073
108. Avet-Loiseau H, Attal M, Moreau P et al (2007) Genetic abnormalities and survival in multiple myeloma: the experience of the Intergroupe Francophone du Myelome. Blood 109(8):3489–3495
109. Avet-Loiseau H, Brigaudeau C, Morineau N et al (1999) High incidence of cryptic translocations involving the Ig heavy chain gene in multiple myeloma, as shown by fluorescence in situ hybridization. Genes Chromosomes Cancer 24(1):9–15
110. Dreyfuss JL, Regatieri CV, Jarrouge TR, Cavalheiro RP, Sampaio LO, Nader HB (2009) Heparan sulfate proteoglycans: structure, protein interactions and cell signaling. An Acad Bras Cienc 81(3):409–429
111. Mackay F, Schneider P, Rennert P, Browning J (2003) BAFF AND APRIL: a tutorial on B cell survival. Annu Rev Immunol 21:231–264
112. Huard B, McKee T, Bosshard C et al (2008) APRIL secreted by neutrophils binds to heparan sulfate proteoglycans to create plasma cell niches in human mucosa. J Clin Invest 118(8):2887–2895
113. Mackay F, Schneider P (2009) Cracking the BAFF code. Nat Rev Immunol 9(7):491–502
114. Moreaux J, Sprynski AC, Dillon SR et al (2009) APRIL and TACI interact with syndecan-1 on the surface of multiple myeloma cells to form an essential survival loop. Eur J Haematol 83(2):119–129
115. Novak AJ, Darce JR, Arendt BK et al (2004) Expression of BCMA, TACI, and BAFF-R in multiple myeloma: a mechanism for growth and survival. Blood 103(2):689–694
116. Tai YT, Li XF, Breitkreutz I et al (2006) Role of B-cell-activating factor in adhesion and growth of human multiple myeloma cells in the bone marrow microenvironment. Cancer Res 66(13):6675–6682
117. Annunziata CM, Davis RE, Demchenko Y et al (2007) Frequent engagement of the classical and alternative NF-kappaB pathways by diverse genetic abnormalities in multiple myeloma. Cancer Cell 12(2):115–130
118. Keats JJ, Fonseca R, Chesi M et al (2007) Promiscuous mutations activate the noncanonical NF-kappaB pathway in multiple myeloma. Cancer Cell 12(2):131–144
119. Demchenko YN, Glebov OK, Zingone A, Keats JJ, Bergsagel PL, Kuehl WM (2010) Classical and/or alternative NF{kappa}B pathway activation in multiple myeloma. Blood 115(17):3541–3552
120. Klein B (2010) Positioning NK-kappaB in multiple myeloma. Blood 115(17):3422–3424
121. Hendriks J, Planelles L, de Jong-Odding J et al (2005) Heparan sulfate proteoglycan binding promotes APRIL-induced tumor cell proliferation. Cell Death Differ 12(6):637–648
122. Ingold K, Zumsteg A, Tardivel A et al (2005) Identification of proteoglycans as the APRIL-specific binding partners. J Exp Med 201(9):1375–1383
123. Moreaux J, Hose D, Jourdan M et al (2007) TACI expression is associated with a mature bone marrow plasma cell signature and C-MAF overexpression in human myeloma cell lines. Haematologica 92(6):803–811
124. Rossi JF, Moreaux J, Hose D et al (2009) Atacicept in relapsed/refractory multiple myeloma or active Waldenstrom's macroglobulinemia: a phase I study. Br J Cancer 101(7): 1051–1058
125. Yaccoby S, Dillon SR, Ling W et al (2006) Atacicept (TACI-Ig) inhibits growth of TACIhigh primary myeloma cells in SCID-hu mice. ASH Annu Meet Abstr 108(11):842
126. Radtke F, Raj K (2003) The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nat Rev Cancer 3(10):756–767
127. Dang TP, Gazdar AF, Virmani AK et al (2000) Chromosome 19 translocation, overexpression of Notch3, and human lung cancer. J Natl Cancer Inst 92(16):1355–1357

128. Bocchetta M, Miele L, Pass HI, Carbone M (2003) Notch-1 induction, a novel activity of SV40 required for growth of SV40-transformed human mesothelial cells. Oncogene 22(1):81–89
129. Rae FK, Stephenson SA, Nicol DL, Clements JA (2000) Novel association of a diverse range of genes with renal cell carcinoma as identified by differential display. Int J Cancer 88(5):726–732
130. Nefedova Y, Cheng P, Alsina M, Dalton WS, Gabrilovich DI (2004) Involvement of Notch-1 signaling in bone marrow stroma-mediated de novo drug resistance of myeloma and other malignant lymphoid cell lines. Blood 103(9):3503–3510
131. Houde C, Li Y, Song L et al (2004) Overexpression of the NOTCH ligand JAG2 in malignant plasma cells from multiple myeloma patients and cell lines. Blood 104(12):3697–3704
132. Jundt F, Probsting KS, Anagnostopoulos I et al (2004) Jagged1-induced Notch signaling drives proliferation of multiple myeloma cells. Blood 103(9):3511–3515
133. Nefedova Y, Sullivan DM, Bolick SC, Dalton WS, Gabrilovich DI (2008) Inhibition of Notch signaling induces apoptosis of myeloma cells and enhances sensitivity to chemotherapy. Blood 111(4):2220–2229
134. Li M, Chen F, Clifton N et al (2010) Combined inhibition of Notch signaling and Bcl-2/Bcl-xL results in synergistic antimyeloma effect. Mol Cancer Ther 9(12):3200–3209
135. Schwarzer R, Kaiser M, Acikgoez O et al (2008) Notch inhibition blocks multiple myeloma cell-induced osteoclast activation. Leukemia 22(12):2273–2277
136. Derksen PW, Tjin E, Meijer HP et al (2004) Illegitimate WNT signaling promotes proliferation of multiple myeloma cells. Proc Natl Acad Sci USA 101(16):6122–6127
137. Qiang YW, Walsh K, Yao L et al (2005) Wnts induce migration and invasion of myeloma plasma cells. Blood 106(5):1786–1793
138. Moon RT, Kohn AD, De Ferrari GV, Kaykas A (2004) WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet 5(9):691–701
139. Tian E, Zhan F, Walker R et al (2003) The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. N Engl J Med 349(26):2483–2494
140. Lu ZY, Brailly H, Rossi JF, Wijdenes J, Bataille R, Klein B (1993) Overall interleukin-6 production exceeds 7 mg/day in multiple myeloma complicated by sepsis. Cytokine 5(6):578–582
141. Condomines M, Veyrune JL, Larroque M et al (2010) Increased plasma-immune cytokines throughout the high-dose melphalan-induced lymphodepletion in patients with multiple myeloma: a window for adoptive immunotherapy. J Immunol 184(2):1079–1084
142. Rossi JF, Fegueux N, Lu ZY et al (2005) Optimizing the use of anti-interleukin-6 monoclonal antibody with dexamethasone and 140 mg/m2 of melphalan in multiple myeloma: results of a pilot study including biological aspects. Bone Marrow Transplant 36(9):771–779
143. Reijmers RM, Groen RW, Rozemuller H et al (2010) Targeting EXT1 reveals a crucial role for heparan sulfate in the growth of multiple myeloma. Blood 115(3):601–604

Chapter 5
Role of Wnt Signaling Pathways in Multiple Myeloma Pathogenesis

Mariateresa Fulciniti and Daniel R. Carrasco

Abstract Multiple myeloma (MM) is a complex and still incurable disease which strongly relies on a network of humoral and cellular interactions within the human bone marrow milieu. The canonical Wnt/b-catenin and the alternative Wnt/RhoA-signaling pathways play important roles in the tropism between MM cells and BM microenvironment, and they have recently been implicated in MM pathogenesis and development of MM bone disease. However, their precise role in growth and survival of myeloma cells remains controversial and needs further investigation. We here summarize the most recent updates of the Wnt/β-catenin signaling pathway in myeloma, and discuss how its various components contribute to MM pathogenesis and related bone disease.

Multiple myeloma (MM) is a neoplasm of plasma cells (PCs), which infiltrate the bone marrow (BM), ultimately leading to pancytopenia and osteolytic bone destruction. The disease represents one of the most common hematological malignancies in adults, and despite recent advances in its treatment, it is still incurable, with a median survival of only 6 years [1], highlighting the need for novel and more effective treatments.

M. Fulciniti
Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Dana 530C, Boston, MA 02115, USA

Jerome Lipper Multiple Myeloma Center, Dana-Farber Cancer Institute, Harvard Medical School,Boston, MA 02115, USA

D.R. Carrasco (✉)
Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Dana 530C, Boston, MA 02115, USA

Department of Pathology, Brigham and Women's Hospital, Boston, MA 02115, USA
e-mail: ruben_carrasco@dfci.harvard.edu

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_5,

MM is typically preceded by an age-progressive condition termed monoclonal gammopathy of undetermined significance (MGUS), which progresses to malignant MM at a rate of 0.5–3% annually [2]. The transition of a PC to a fully transformed aggressive myeloma is a multistep process, which requires the acquisition of mutations in several protooncogenes and tumor suppressor genes [3, 4], as well as alterations in many signaling transduction pathways including the canonical Wnt pathway [5].

Most of MM disease evolution takes place in the BM. There is now an increased understanding of how the adhesion of MM cells to BM further affects gene expression in tumor cells and in bone marrow stromal cells (BMSCs), thereby increasing tumor growth, survival, drug resistance, and migration. The biological and clinical behavior of MM cells is therefore not exclusively determined by their intrinsic genetic profile, but is also influenced by intricate bidirectional interactions with their local bone microenvironment, which constitutes a sanctuary for MM cells [6]. As a result of these advances, a new treatment paradigm has emerged in MM, which is based on the concurrent targeting of both the tumor cell and its BM milieu to overcome drug resistance and improve patient outcome.

While substantial progress has been made in characterizing the bidirectional interactions between MM cells and their bone microenvironment, several fundamental questions remain unanswered. For instance, it is not clear yet which of these intercellular interactions have the highest contribution to the protective effects of the local milieu on MM cells and which specific molecular pathways mediate the biologic effects of MM–BM microenvironment interactions.

The canonical Wnt/β-catenin and the alternative Wnt/RhoA-signaling pathways play important roles in the tropism between MM cells and BM microenvironment, and they have recently been implicated in MM pathogenesis [7]. Conversely, other reports have shown the production by MM cells of soluble inhibitors of Wnt signaling, such as Dickkopf-1 (DKK1) and secreted frizzled-related protein-3 (sFRP3), which cause MM bone disease [8, 9]. However, the precise role of Wnt pathway in growth and survival of myeloma cells remains unclear and often controversial. A complete understanding of the complex molecular genetics of β-catenin activity in MM would therefore provide valuable insight and could help to develop new therapies in MM and other malignancies with aberrant Wnt/β-catenin activity [10].

5.1 The Wnt Signaling Pathway

Wnts comprise a family of glycoproteins critical for normal development [11, 12], which have been also linked to several forms of cancer [13], including hematologic malignancies [7]. WNT genes encode a family of 19 secreted glycoproteins, which promiscuously interact with several transmembrane Frizzled receptors and the low-density lipoprotein receptor-related protein (LRP) 5/6 [14]. This interaction leads to the activation of downstream elements known as Dishevelleds (Dvls) [15] and

subsequently a number of intracellular signaling cascades that control gene expression, cell behavior, cell adhesion, and cell polarity, during both embryonic development and postnatal life [11, 12, 16]. The best studied of these signaling pathway is referred to as the "canonical" (Wnt/β-catenin) pathway, in which the key event is the stabilization of β-catenin. β-catenin plays two important roles in cells, as transcriptional activator in Wnt signaling [17, 18] and as protein associated with E-cadherin in cell–cell adhesion [19].

In the absence of a Wnt signal, the cytoplasmic β-catenin interacts with a multicomponent complex consisting of the tumor suppressor gene product adenomatous polyposis coli (APC), Axin scaffold proteins, glycogen synthase kinase-3β (GSK3β), and casein kinase 1α, which promotes the phosphorylation of specific serine and threonine residues in the N-terminal region of β-catenin. This GSK3β-mediated phosphorylation marks β-catenin for degradation by the ubiquitin–proteasome pathway [20]. Levels of free β-catenin in the cell consequently remain low, and formation of active nuclear Tcf/β-catenin transcriptional complexes is decreased.

Upon Wnt stimulation, however, the kinase complex is dissociated, leading to the accumulation of active nonphosphorylated form of β-catenin in the cytoplasm and its further translocation to the nucleus. Here, it interacts with members of the lymphoid enhancer factor (LEF)/T-cell factor (TCF) family of transcription factors [21, 22] driving the transcription of target genes like c-Myc, cyclin D1, Tcf-1, and Axin2, which are involved in cell proliferation, migration, and survival [23–25].

Several components of the canonical Wnt signaling cascade have been shown to function as either tumor suppressor genes or as oncogenes in a wide range of common human cancers [13, 26, 27]. Recent studies have revealed that high Wnt signaling activity functionally designates the cancer stem cell population, underscoring the relevance of this pathway for target drug discovery and therapeutic development [10, 13, 17, 28]. Inactivating mutations of the tumor suppressor genes APC and Axin or activating mutations of the sequences encoding the crucial GSK3β phosphorylation sites in the N-terminal domain of β-catenin have been found in the majority of colorectal cancers, as well as many other cancer types. The critical consequence of these mutations is the elevation of nuclear β-catenin, leading to the formation of constitutive nuclear β-catenin/TCF complexes and altered expression of TCF target genes known to cooperate in neoplastic transformation such as CCND1 (cyclin D1), MYC, and CD44 [29].

In addition to the canonical pathway, Wnts initiate a second cascade (Wnt/RhoA) that does not require the LRP coreceptor leading to activation of RhoA and associated downstream kinases [30, 31]. RhoA is a member of a family of small guanosine triphosphatases (GTPases) that includes Rac and Cdc42 [32]. This pathway has been implicated in cell motility and adhesion [33], and several cell types have been shown to respond with changes in these properties in response to a variety of Wnts [34, 35].

Finally, activation of PKCs has been associated with a third Wnt signaling pathway characterized by calcium flux and likely involving G protein-coupled receptors [36, 37].

5.2 Role of WNT Pathway in MM Cell Growth and Survival

Constitutively active canonical Wnt/β-catenin pathway has been documented in myeloma cell lines and malignant PCs from patients with MM [9, 38]. Specifically, it is the N-terminally unphosphorylated β-catenin to be overexpressed in MM, and the cause for this constitutive accumulation in myeloma cells has not been determined yet. To note, no mutations in the Wnt pathway have been identified in myeloma cells, suggesting that mechanisms other than gene mutations may contribute to Wnt pathway deregulation in myeloma [7, 38, 39]. A recent report provided evidence that deregulation of BCL9, known to play an important role in transcriptional activity of β-catenin in association with LEF/TCF family members [40], can be one alternative pathway for β-catenin activation [41].

The precise role of Wnt in growth and survival of myeloma cells is intriguing and still controversial. On one hand and in agreement with the general notion that activating mutation in the canonical Wnt pathway promotes tumor growth, there are several reports along these lines in MM. For instance, Derksen et al. demonstrated that stimulation of the canonical Wnt signaling pathway with Wnt3a additionally increases both accumulation and nuclear localization of β-catenin, leading to enhanced MM cell proliferation [38]. In addition, the small molecule PKF115-584, recently identified by high-throughput ELISA screening, efficiently blocks the formation of the β-catenin/TCF transcriptional complex and thereby expression of Wnt target genes inducing cytotoxicity in both patient MM cells and MM cell lines [39]. Furthermore, Dutta-Simmons and colleagues have shown β-catenin accumulation in MM primary tumors compared with normal plasma cells and provided evidence for a novel functional link between β-catenin and Aurora kinase A, underscoring a critical role of these pathways in MM disease progression [42]. Importantly, they showed that a decrease in β-catenin protein caused significant tumor remission and increased survival in a mouse xenograft model of MM. Since the Wnt/β-catenin pathway is critical for normal cellular functions like hematopoietic stem cell homeostasis, inhibition of the entire pathway would have undesired effects [26, 43, 44]. Therefore, interference with specific interactions of the β-catenin transcriptional complex such as that between β-catenin and BCL9, which reduce only the expression of selected target genes, could be a good therapeutic option. A β-catenin small-interfering RNA treatment inhibited myeloma cell growth in vivo in a xenograft model of myeloma [45]. Additional strategies to target Wnt signaling pathways include the use of small-molecule inhibitors, which block interaction of β-catenin with CREB-binding protein, siRNAs, and antibodies directed against WNTs [46]. Indeed, constitutively, activation of Wnt signaling correlates with hypermethylation and hence silencing of Wnt antagonist genes. Consequently, demethylation of methylated Wnt inhibitors downregulates Wnt signaling and associated MM cell proliferation [47].

In contrast, other studies have shown that the activation of canonical Wnt signaling is not associated with proliferative growth effect, despite the increase in β-catenin activity. Moreover, activation of canonical Wnt signaling by Wnt3a in a SCID-hu model of myeloma resulted in cell growth inhibition when growth was restricted to implanted human bones in SCID mice and not when the tumor was growing subcutaneously [48].

A recent report showed that LiCl, a putative β-catenin activator, could suppress MM growth and bone disease in the 5TGM model of myeloma, while promote proliferation in a subcutaneous model of myeloma [49]. These reports suggest the role of the canonical Wnt pathway in the context of the myeloma microenvironment and also confirm the need to study MM in vivo using animal models that take into consideration the influences of the human bone milieu. Consistent with these data, Fulciniti et al. have also shown that BHQ880, a human DKK1 neutralizing antibody, has been shown to increase function and number of osteoblasts (OBs) and to induce activation of β-catenin and downregulation of NF-κB activity in BMSC along with anti-MM effect when evaluated in the presence of the BM milieu [8]. In addition, GSK-3β inhibition has been shown to decrease MM cell growth despite the increased β-catenin levels, ameliorate the bone destruction in a murine model of myeloma bone disease [50], and augment the response to the cytotoxic effects of bortezomib, a clinically used chief therapeutic agent in MM therapy [51].

Emerging data support an important role for Wnt/β-catenin activation as a mediator of chemoresistance and induction of cell adhesion-mediated drug resistance (CAM-DR) in hepatocellular carcinoma [52], laryngeal carcinoma [53], neuroblastomas [54], and acute myeloid leukemia [55]. In MM, Wnt signaling pathways have been implicated in the induction of CAM-DR through integrin α6β1 (VLA-6) and RhoA-Rho kinase-signaling sequelae [56]. A recent paper has reported that standard care therapy for MM, lenalidomide, induces Wnt/β-catenin pathway activation in myeloma cells [57], unlike thalidomide, which inhibits Wnt/β-catenin. Stimulation of Wnt/β-catenin signaling with recombinant Wnt-3a, or by overexpression of β-catenin, reduced the antiproliferative activity of lenalidomide. Conversely, suppression of β-catenin with small hairpin RNAs restored myeloma cell sensitivity to lenalidomide. Together, these findings support the hypothesis that lenalidomide-mediated activation of Wnt/β-catenin signaling in myeloma cells may contribute to mechanisms of resistance to this agent.

Finally, previous studies have examined the role of Wnt pathway in migration, which is one of the important processes fundamental to myeloma cell invasion and dissemination. These studies have demonstrated that activation of the Wnt/RhoA pathway, which does not require the LRP coreceptor, induces striking morphologic changes in myeloma cells, suggesting altered motility and making Wnts likely candidates as participants in the migratory process [58].

5.3 Role of WNT Pathway in MM Bone Disease

A cardinal clinical feature of MM is the presence of osteolytic bone lesions. Under physiological conditions, the skeleton undergoes constant structural remodeling in order to optimize the stress-bearing capacity of the bones. This physiologic process consists of precisely coordinated cycles of osteoclast (OC)-mediated resorption of old bone and subsequent compensatory formation of new bone by OB. In MM, however, these two functionally opposing process are uncoupled [59, 60]. Various clinical observations [61] and experimental studies [62, 63] have linked the level of MM bone

disease with disease burden. There is also emerging evidence that the cellular bone compartment affects MM cell growth and progression, supported by the observation that OC can sustain long-term survival and proliferation of primary MM cells [64, 65] while OB may impede MM cell growth [66, 67]. Therefore, developing novel therapies that are able to target both tumor cells and bone disease is one of the major goals of the treatment for this disease. Indeed, it has been shown that many novel biologic agents being used for the treatment of MM also further inhibit the bone destructive process (e.g., bortezomib, IMID drugs, SDX-308) [68–71].

Increased osteoclastic activity and its molecular basis have long been considered a primary pathogenic event in MM bone disease. However, a molecular basis for the well-recognized lack of OB function in the MM bone disease has only recently been described [66, 72]. Canonical Wnt pathway plays an important role in controlling proliferation, differentiation, and survival of OBs [73–76]. Increasing data suggest a role for Wnt signaling pathway in the development of myeloma bone disease, and recent published data also linked Wnt/β-catenin signaling with osteoclastogenesis in MM [77].

Human genetic bone diseases and in vivo mouse models provide strong evidence for the function of the Wnt signaling pathway in bone biology. Inactivating mutations in the gene for LRP5 result in osteoporosis-pseudoglioma syndrome in humans, whereas "gain of function" mutations in LRP5 are associated with a syndrome of hereditary high bone density [74, 78]. Overexpression of β-catenin in OBs has been demonstrated to induce a high bone mass phenotype [79]. Transgenic mice overexpressing DKK1, in OBs develop severe osteopenia, whereas deletion of a single allele of DKK1 caused an increase in bone mass [80, 81].

Preclinical studies have demonstrated that treatment of myelomatous SCID-hu mice, carrying primary disease, with recombinant Wnt3a stimulated bone formation [48]. On the other hand, the proteasome-inhibitor bortezomib promotes matrix mineralization and calcium deposition by osteoprogenitor cells and primary mesenchymal stem cells via Wnt-independent activation of beta-catenin/TCF signaling [82].

Tian et al. reported the production of DKK1 by primary CD138+ MM cells, but not by plasma cells from MGUS patients and showed the correlation of the levels of DKK1 mRNA with focal bone lesions in patients with MM [83]. Politou et al. [84] have confirmed the increased DKK1 levels in the sera of MM patients, and Kaiser et al. [85] have reported that serum DKK1 levels correlate with the extent of bone disease in MM patients. The DKK1 produced by MM cells can inhibit the differentiation of OB precursor cells [83] and bone formation in vitro [9] through a DKK1-mediated attenuation of Wnt3a-induced stabilization of β-catenin [86]. These findings confirm DKK1 as an important regulator of bone formation in the bone microenvironment. Production of DKK1 is also modulated by a number of drugs used to treat MM including dexamethasone [87] and the immunomodulatory agents, thalidomide and lenalidomide [88, 89]. The treatment of OB with dexamethasone results in a time- and dose-dependent increase in DKK1 expression, suggesting that dexamethasone also blocks bone formation through a mechanism affecting osteoblastogenesis providing partial explanation for osteoporosis observed

with long-term glucocorticoid use [87]. Thus, combining current treatments with anti-DKK1 antibody may abrogate dexamethasone-induced osteoporosis and stimulate bone formation. Yaccoby et al. have previously shown that daily injections of a neutralizing DKK1 antibody in the area surrounding myelomatous bone ameliorated bone turnover and reduced tumor burden in the SCID-rab model [90]. Moreover, a human DKK1 neutralizing antibody has been shown to increase function and number of OB in a SCID-hu model of MM in which human myeloma cell growth is restricted to human bone implanted into SCID mice [8]. Edwards et al. demonstrated that increasing Wnt signaling within the myeloma bone microenvironment can inhibit the development of myeloma bone disease, while increase myeloma growth at non-osseous sites [49], raising clinical concerns for targeting Wnt signaling as a therapeutic approach in myeloma due to the risk of increasing tumor growth outside the BM microenvironment.

Furthermore, myeloma cell lines and primary myeloma cells from patients with bone lesions have been shown to produce the soluble Wnt inhibitor sFRP-2 and thereby suppress mineralization and alkaline phosphatase activity in OBs. Immunodepletion of sFRP-2 significantly restored mineralized nodule formation in vitro [91].

Overall, these antecedents suggest the critical role of Wnt signaling pathway in the pathogenesis of myeloma bone disease.

5.4 Conclusions

Despite the advances in our understanding of the biology of MM, yet a number of critical questions remain unanswered and myeloma remains an incurable malignancy. Increasing evidences suggest the link between Wnt pathway and the development of MM bone disease. The discovery of Dkk1 as a mediator of OB dysfunction in myeloma identifies the Wnt signaling pathway as a potential therapeutic target for the treatment of myeloma bone disease. However, its precise role in growth and survival of myeloma cells remains controversial and needs further investigation due to the clinical concerns of increasing tumor growth while targeting Wnt signaling as a therapeutic approach in myeloma bone disease. The role of the Wnt signaling pathway in myeloma cells may be more complex and dependent upon the myeloma microenvironment and the local balance of agonists and antagonists of this pathway, suggesting the need to study this disease in vivo taken into consideration the influences of the bone milieu.

References

1. Rajkumar SV, Kyle RA (2005) Multiple myeloma: diagnosis and treatment. Mayo Clin Proc 80:1371–1382
2. Mitsiades CS, Mitsiades N, Munshi NC, Anderson KC (2004) Focus on multiple myeloma. Cancer Cell 6:439–444

3. Kuehl WM, Bergsagel PL (2002) Multiple myeloma: evolving genetic events and host interactions. Nat Rev Cancer 2:175–187
4. Carrasco DR, Tonon G, Huang Y et al (2006) High-resolution genomic profiles define distinct clinico-pathogenetic subgroups of multiple myeloma patients. Cancer Cell 9:313–325
5. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC (2007) Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. Nat Rev Cancer 7:585–598
6. Podar K, Chauhan D, Anderson KC (2009) Bone marrow microenvironment and the identification of new targets for myeloma therapy. Leukemia 23:10–24
7. Qiang YW, Endo Y, Rubin JS, Rudikoff S (2003) Wnt signaling in B-cell neoplasia. Oncogene 22:1536–1545
8. Fulciniti M, Tassone P, Hideshima T et al (2009) Anti-DKK1 mAb (BHQ880) as a potential therapeutic agent for multiple myeloma. Blood 114:371–379
9. Giuliani N, Morandi F, Tagliaferri S et al (2007) Production of Wnt inhibitors by myeloma cells: potential effects on canonical Wnt pathway in the bone microenvironment. Cancer Res 67:7665–7674
10. Barker N, Clevers H (2006) Mining the Wnt pathway for cancer therapeutics. Nat Rev Drug Discov 5:997–1014
11. Wodarz A, Nusse R (1998) Mechanisms of Wnt signaling in development. Annu Rev Cell Dev Biol 14:59–88
12. Veeman MT, Axelrod JD, Moon RT (2003) A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Dev Cell 5:367–377
13. Logan CY, Nusse R (2004) The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20:781–810
14. He X, Semenov M, Tamai K, Zeng X (2004) LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. Development 131:1663–1677
15. Wharton KA Jr (2003) Runnin' with the Dvl: proteins that associate with Dsh/Dvl and their significance to Wnt signal transduction. Dev Biol 253:1–17
16. Moon RT, Bowerman B, Boutros M, Perrimon N (2002) The promise and perils of Wnt signaling through beta-catenin. Science 296:1644–1646
17. Polakis P (2000) Wnt signaling and cancer. Genes Dev 14:1837–1851
18. Willert K, Nusse R (1998) Beta-catenin: a key mediator of Wnt signaling. Curr Opin Genet Dev 8:95–102
19. Ben-Ze'ev A, Geiger B (1998) Differential molecular interactions of beta-catenin and plakoglobin in adhesion, signaling and cancer. Curr Opin Cell Biol 10:629–639
20. Aberle H, Bauer A, Stappert J, Kispert A, Kemler R (1997) beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J 16:3797–3804
21. Molenaar M, van de Wetering M, Oosterwegel M et al (1996) XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. Cell 86:391–399
22. Behrens J, von Kries JP, Kuhl M et al (1996) Functional interaction of beta-catenin with the transcription factor LEF-1. Nature 382:638–642
23. He TC, Sparks AB, Rago C et al (1998) Identification of c-MYC as a target of the APC pathway. Science 281:1509–1512
24. Shtutman M, Zhurinsky J, Simcha I et al (1999) The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci USA 96:5522–5527
25. Roose J, Huls G, van Beest M et al (1999) Synergy between tumor suppressor APC and the beta-catenin-Tcf4 target Tcf1. Science 285:1923–1926
26. Staal FJ, Clevers HC (2005) WNT signalling and haematopoiesis: a WNT-WNT situation. Nat Rev Immunol 5:21–30
27. Clevers H (2006) Wnt/beta-catenin signaling in development and disease. Cell 127:469–480
28. Vermeulen L, De Sousa EMF, van der Heijden M et al (2010) Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol 12:468–476
29. Wielenga VJ, Smits R, Korinek V et al (1999) Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. Am J Pathol 154:515–523

30. Strutt DI, Weber U, Mlodzik M (1997) The role of RhoA in tissue polarity and Frizzled signalling. Nature 387:292–295
31. Winter CG, Wang B, Ballew A et al (2001) Drosophila Rho-associated kinase (Drok) links Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. Cell 105:81–91
32. Ridley AJ, Hall A (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70:389–399
33. Nelson WJ, Nusse R (2004) Convergence of Wnt, beta-catenin, and cadherin pathways. Science 303:1483–1487
34. Weeraratna AT, Jiang Y, Hostetter G et al (2002) Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. Cancer Cell 1:279–288
35. Ouko L, Ziegler TR, Gu LH, Eisenberg LM, Yang VW (2004) Wnt11 signaling promotes proliferation, transformation, and migration of IEC6 intestinal epithelial cells. J Biol Chem 279:26707–26715
36. Kuhl M, Sheldahl LC, Park M, Miller JR, Moon RT (2000) The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape. Trends Genet 16:279–283
37. Wang HY, Malbon CC (2003) Wnt signaling, Ca2+, and cyclic GMP: visualizing Frizzled functions. Science 300:1529–1530
38. Derksen PW, Tjin E, Meijer HP et al (2004) Illegitimate WNT signaling promotes proliferation of multiple myeloma cells. Proc Natl Acad Sci USA 101:6122–6127
39. Sukhdeo K, Mani M, Zhang Y et al (2007) Targeting the beta-catenin/TCF transcriptional complex in the treatment of multiple myeloma. Proc Natl Acad Sci USA 104:7516–7521
40. Kramps T, Peter O, Brunner E et al (2002) Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. Cell 109:47–60
41. Mani M, Carrasco DE, Zhang Y et al (2009) BCL9 promotes tumor progression by conferring enhanced proliferative, metastatic, and angiogenic properties to cancer cells. Cancer Res 69:7577–7586
42. Dutta-Simmons J, Zhang Y, Gorgun G et al (2009) Aurora kinase A is a target of Wnt/beta-catenin involved in multiple myeloma disease progression. Blood 114:2699–2708
43. Reya T, Duncan AW, Ailles L et al (2003) A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 423:409–414
44. van de Wetering M, de Lau W, Clevers H (2002) WNT signaling and lymphocyte development. Cell 109(Suppl):S13–S19
45. Ashihara E, Kawata E, Nakagawa Y et al (2009) beta-catenin small interfering RNA successfully suppressed progression of multiple myeloma in a mouse model. Clin Cancer Res 15:2731–2738
46. Lepourcelet M, Chen YN, France DS et al (2004) Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex. Cancer Cell 5:91–102
47. Chim CS, Pang R, Fung TK, Choi CL, Liang R (2007) Epigenetic dysregulation of Wnt signaling pathway in multiple myeloma. Leukemia 21:2527–2536
48. Qiang YW, Shaughnessy JD Jr, Yaccoby S (2008) Wnt3a signaling within bone inhibits multiple myeloma bone disease and tumor growth. Blood 112:374–382
49. Edwards CM, Edwards JR, Lwin ST et al (2008) Increasing Wnt signaling in the bone marrow microenvironment inhibits the development of myeloma bone disease and reduces tumor burden in bone in vivo. Blood 111:2833–2842
50. Gunn WG, Krause U, Lee N, Gregory CA (2011) Pharmaceutical inhibition of glycogen-synthetase-kinase-3-beta reduces multiple myeloma-induced bone disease in a novel murine plasmacytoma xenograft model. Blood 117(5):1641–1651
51. Piazza F, Manni S, Tubi LQ et al (2010) Glycogen Synthase Kinase-3 regulates multiple myeloma cell growth and bortezomib-induced cell death. BMC Cancer 10:526
52. Noda T, Nagano H, Takemasa I et al (2009) Activation of Wnt/beta-catenin signalling pathway induces chemoresistance to interferon-alpha/5-fluorouracil combination therapy for hepatocellular carcinoma. Br J Cancer 100:1647–1658
53. Cimbora-Zovko T, Ambriovic-Ristov A, Loncarek J, Osmak M (2007) Altered cell-cell adhesion in cisplatin-resistant human carcinoma cells: a link between beta-catenin/plakoglobin ratio and cisplatin resistance. Eur J Pharmacol 558:27–36

54. Flahaut M, Meier R, Coulon A et al (2009) The Wnt receptor FZD1 mediates chemoresistance in neuroblastoma through activation of the Wnt/beta-catenin pathway. Oncogene 28:2245–2256
55. De Toni F, Racaud-Sultan C, Chicanne G et al (2006) A crosstalk between the Wnt and the adhesion-dependent signaling pathways governs the chemosensitivity of acute myeloid leukemia. Oncogene 25:3113–3122
56. Kobune M, Chiba H, Kato J et al (2007) Wnt3/RhoA/ROCK signaling pathway is involved in adhesion-mediated drug resistance of multiple myeloma in an autocrine mechanism. Mol Cancer Ther 6:1774–1784
57. Bjorklund CC, Ma W, Wang ZQ et al (2011) Evidence of a role for activation of Wnt/{beta}-catenin signaling in the resistance of plasma cells to lenalidomide. J Biol Chem 286(13):11009–11020
58. Qiang YW, Walsh K, Yao L et al (2005) Wnts induce migration and invasion of myeloma plasma cells. Blood 106:1786–1793
59. Roodman GD (2004) Pathogenesis of myeloma bone disease. Blood Cells Mol Dis 32:290–292
60. Sezer O (2005) Myeloma bone disease. Hematology 10(Suppl 1):19–24
61. Coleman RE, Major P, Lipton A et al (2005) Predictive value of bone resorption and formation markers in cancer patients with bone metastases receiving the bisphosphonate zoledronic acid. J Clin Oncol 23:4925–4935
62. Pearse RN, Sordillo EM, Yaccoby S et al (2001) Multiple myeloma disrupts the TRANCE/osteoprotegerin cytokine axis to trigger bone destruction and promote tumor progression. Proc Natl Acad Sci USA 98:11581–11586
63. Vanderkerken K, De Leenheer E, Shipman C et al (2003) Recombinant osteoprotegerin decreases tumor burden and increases survival in a murine model of multiple myeloma. Cancer Res 63:287–289
64. Abe M, Hiura K, Wilde J et al (2004) Osteoclasts enhance myeloma cell growth and survival via cell-cell contact: a vicious cycle between bone destruction and myeloma expansion. Blood 104:2484–2491
65. Yaccoby S, Wezeman MJ, Henderson A et al (2004) Cancer and the microenvironment: myeloma-osteoclast interactions as a model. Cancer Res 64:2016–2023
66. Yaccoby S, Wezeman MJ, Zangari M et al (2006) Inhibitory effects of osteoblasts and increased bone formation on myeloma in novel culture systems and a myelomatous mouse model. Haematologica 91:192–199
67. Li X, Pennisi A, Yaccoby S (2008) Role of decorin in the antimyeloma effects of osteoblasts. Blood 112:159–168
68. Mukherjee S, Raje N, Schoonmaker JA et al (2008) Pharmacologic targeting of a stem/progenitor population in vivo is associated with enhanced bone regeneration in mice. J Clin Invest 118:491–504
69. Giuliani N, Rizzoli V, Roodman GD (2006) Multiple myeloma bone disease: pathophysiology of osteoblast inhibition. Blood 108:3992–3996
70. Feng R, Anderson G, Xiao G et al (2007) SDX-308, a nonsteroidal anti-inflammatory agent, inhibits NF-kappaB activity, resulting in strong inhibition of osteoclast formation/activity and multiple myeloma cell growth. Blood 109:2130–2138
71. Anderson G, Gries M, Kurihara N et al (2006) Thalidomide derivative CC-4047 inhibits osteoclast formation by down-regulation of PU.1. Blood 107:3098–3105
72. Stewart JP, Shaughnessy JD Jr (2006) Role of osteoblast suppression in multiple myeloma. J Cell Biochem 98:1–13
73. Baron R, Rawadi G, Roman-Roman S (2006) Wnt signaling: a key regulator of bone mass. Curr Top Dev Biol 76:103–127
74. Gong Y, Slee RB, Fukai N et al (2001) LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell 107:513–523
75. Kato M, Patel MS, Levasseur R et al (2002) Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. J Cell Biol 157:303–314

76. Kulkarni NH, Onyia JE, Zeng Q et al (2006) Orally bioavailable GSK-3alpha/beta dual inhibitor increases markers of cellular differentiation in vitro and bone mass in vivo. J Bone Miner Res 21:910–920
77. Qiang YW, Chen Y, Brown N et al (2010) Characterization of Wnt/beta-catenin signalling in osteoclasts in multiple myeloma. Br J Haematol 148:726–738
78. Boyden LM, Mao J, Belsky J et al (2002) High bone density due to a mutation in LDL-receptor-related protein 5. N Engl J Med 346:1513–1521
79. Glass DA II, Bialek P, Ahn JD et al (2005) Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. Dev Cell 8:751–764
80. Li J, Sarosi I, Cattley RC et al (2006) Dkk1-mediated inhibition of Wnt signaling in bone results in osteopenia. Bone 39:754–766
81. Morvan F, Boulukos K, Clement-Lacroix P et al (2006) Deletion of a single allele of the Dkk1 gene leads to an increase in bone formation and bone mass. J Bone Miner Res 21:934–945
82. Qiang YW, Hu B, Chen Y et al (2009) Bortezomib induces osteoblast differentiation via Wnt-independent activation of beta-catenin/TCF signaling. Blood 113:4319–4330
83. Tian E, Zhan F, Walker R et al (2003) The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. N Engl J Med 349:2483–2494
84. Politou MC, Heath DJ, Rahemtulla A et al (2006) Serum concentrations of Dickkopf-1 protein are increased in patients with multiple myeloma and reduced after autologous stem cell transplantation. Int J Cancer 119:1728–1731
85. Kaiser M, Mieth M, Liebisch P et al (2008) Serum concentrations of DKK-1 correlate with the extent of bone disease in patients with multiple myeloma. Eur J Haematol 80:490–494
86. Qiang YW, Barlogie B, Rudikoff S, Shaughnessy JD Jr (2008) Dkk1-induced inhibition of Wnt signaling in osteoblast differentiation is an underlying mechanism of bone loss in multiple myeloma. Bone 42:669–680
87. Hurson CJ, Butler JS, Keating DT et al (2007) Gene expression analysis in human osteoblasts exposed to dexamethasone identifies altered developmental pathways as putative drivers of osteoporosis. BMC Musculoskelet Disord 8:12
88. Colla S, Zhan F, Xiong W et al (2007) The oxidative stress response regulates DKK1 expression through the JNK signaling cascade in multiple myeloma plasma cells. Blood 109:4470–4477
89. Shaughnessy JD Jr, Barlogie B (2003) Interpreting the molecular biology and clinical behavior of multiple myeloma in the context of global gene expression profiling. Immunol Rev 194:140–163
90. Yaccoby S, Ling W, Zhan F, Walker R, Barlogie B, Shaughnessy JD Jr (2007) Antibody-based inhibition of DKK1 suppresses tumor-induced bone resorption and multiple myeloma growth in vivo. Blood 109:2106–2111
91. Oshima T, Abe M, Asano J et al (2005) Myeloma cells suppress bone formation by secreting a soluble Wnt inhibitor, sFRP-2. Blood 106:3160–3165

Chapter 6
The mTOR Pathway in Multiple Myeloma

Joseph Gera and Alan Lichtenstein

Abstract The mammalian target of rapamycin (mTOR) is centrally located, linking proximal oncogenic cascades to critical downstream pathways that drive tumor growth. MTOR regulates such diverse functions as protein translation, proliferation, viability, autophagy, metabolism homeostasis, monitoring of energy reserves and induction of angiogenesis. Given its fundamental role in tumorigenesis, it is not surprising that a huge effort is being made to develop mTOR inhibitors. The existence of feedback pathways, that become activated subsequent to mTOR inhibition, has complicated these efforts. However, the fact that mTOR exists in two separate complexes, TORC1 and TORC2, that rapalogs primarily inhibit only TORC1 and that TORC2 is actually a key activator of AKT has injected new energy in the quest to find inhibitors that can inhibit both complexes. In myeloma models, pre-clinical studies confirm activity of rapalogs as well as newer TORC1/TORC2 inhibitors and early phase clinical trials have begun. In addition, the recent finding of upregulated myeloma cell expression of DEPTOR, an mTOR binding protein that restricts mTOR activity, suggests an additional future therapeutic target specific to the myeloma tumor model.

J. Gera
Division of Hematology-Oncology, UCLA-Greater Los Angeles VA Healthcare Center, Los Angeles, CA, USA

A. Lichtenstein, M.D. (✉)
Division of Hematology-Oncology, UCLA-Greater Los Angeles VA Healthcare Center, Los Angeles, CA, USA

W111H, West LA VA Medical Center, 11301 Wilshire BLVD, Los Angeles, CA 90073, USA
e-mail: alan.lichtenstein@med.va.gov

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_6,

6.1 Introduction

The mammalian target of rapamycin (mTOR) kinase is critical for tumor cell proliferative, anti-apoptotic and pro-angiogenic activities. As such, it has become a popular molecular target for new therapeutics. The characterization of mTOR followed the previous discovery of a macrolide antibiotic produced by *Streptomyces hygroscopicus*, a streptomycete isolated from soil collected from the Easter Island known as Rapa Nui [1]. This potent antifungal drug was named after its geographical site of origin; hence, rapamycin. Subsequently, TOR proteins were identified during a screen for mutations that induced resistance to rapamycin in budding yeast [2]. TOR homologs were then identified in many species and it is now clear that this serine/threonine kinase has been conserved throughout eukaryotic evolution for the purpose of coordinating cellular growth responses to levels of amino acids, growth factors, and energy capability.

MTOR belongs to the phosphatidylinositol 3-kinase-related family of kinases and functions within two separate multicomponent complexes: TORC1 and TORC2 [3]. These individual complexes have different substrates and regulate distinct cellular responses (see below). As many of these responses are obviously pro-tumorigenic and, since TORC1 and TORC2 activities are upregulated in the majority of malignancies, the development of mTOR inhibitors has become a priority. First generation mTOR inhibitors, including rapamycin and related rapalog compounds such as temsirolimus and everolimus, associate with the FKB12 protein and together they bind to mTOR adjacent to its kinase domain [4]. At that site they allostearically inhibit mTOR, primarily that within the TORC1 complex. Newer second generation mTOR inhibitors specifically inhibit the mTOR kinase domain and significantly suppress both TORC2 as well as TORC1 activity. In this report, we review the molecular biology of mTOR as well as preclinical studies that support use of mTOR inhibitors in myeloma. We also address the myeloma-specific characteristics of mTOR and mTOR inhibitor activity. Finally, we will review the few clinical trials that have been performed.

6.2 Structure of mTOR

The mammalian TOR kinase is a 300 kDa signaling protein founding member of the PIKK family of kinases and consists of several highly conserved domains. These include the HEAT repeats, as well as the FAT, FRB (FKBP12-binding domain), kinase, FIT, and FATC domains (Fig. 6.1). The HEAT repeat regions in the molecule are located in the N-terminal and central portions of the kinase and are believed to function as a protein–protein interaction surface [5]. The HEAT repeats form a large superhelical structure and are required for raptor binding [6] and for localization to

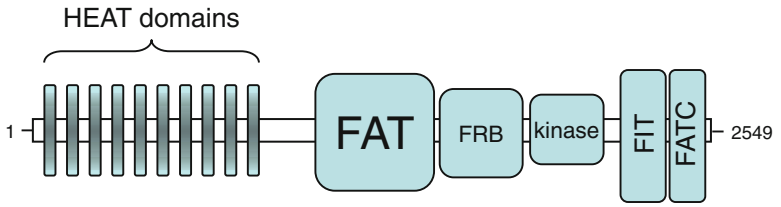


Fig. 6.1 Schematic diagram of mTOR domains. See text for description of the various domain regions

the Golgi and endoplasmic reticulum compartments [7]. A second isoform of mTOR has recently been described, mTORβ, which can promote cell proliferation and tumorigenesis [8]. This isoform lacks the HEAT repeats but is competent to bind both raptor and rictor and can signal to both TORC1 and TORC2 substrates, suggesting that the N-terminal 23 amino acids within mTOR are sufficient to mediate interaction with its protein partners.

The most highly conserved region of mTOR comprises the domains C-terminal to the HEAT repeats. This portion of the molecule contains the FAT, FRB, kinase, FIT, and FATC domains. The FAT domain consists of ~500 amino acids and has been proposed to contain additional HEAT domains possibly involved in protein–protein interactions [9]. The FATC domain is located at the C terminus and is required for mTOR function. Nuclear magnetic resonance studies indicate that a disulfide bridge in the FATC domain may link the cellular redox state to mTOR stability and activity [10].

The FRB domain consists of ~100 amino acids within mTOR and has been proposed to contain an additional pair of HEAT domains [11]. Two missense mutations have been characterized in this domain, originally identified in yeast, which prevent binding of FKBP12-rapamycin to mTOR and confer rapamycin resistance when either of these alleles is overexpressed [11].

The kinase domain of mTOR is ~300 residues in length and the N-terminal portion of this domain contains the binding site for the TORC1 upstream activator Rheb. Both the active (GTP-bound) and inactive (GDP-bound) forms of Rheb are capable of binding mTOR in vitro; however, only the active form can promote TORC1 activity [12]. Recent homology modeling studies suggest that the ATP-binding site in the kinase domain forms a novel pharmacophore region which may give insights as to the efficacy of the second generation mTOR kinase inhibitors currently in clinical development [13].

The newly defined FIT domain lies between the kinase and FATC regions of mTOR [13]. While not highly conserved across many species, the function of this region is consistent with previous observations that the FIT domain may be regulated by reversible phosphorylation and may function as a repressor domain [14–18].

6.3 Architecture of the TORCS and Their Substrate Specificity

The mTOR kinase is incorporated into at least two different complexes in mammalian cells [6, 19]. MTORC1 is sensitive to the macrolide rapamycin while mTORC2 is relatively resistant [20, 21]. The major mTORC1 components include mTOR, Raptor and mLST8/GβL and the mTORC2 components are mTOR, Rictor, and mLST8/GβL. The well-characterized substrates for mTORC1 are p70S6K and 4E-BP-1 while mTORC2 is presently known to directly regulate PKC-α, SGK, and AKT (see Fig. 6.2) [20, 22, 23]. Additionally, there are several accessory binding proteins that have now been characterized which interact with mTOR and regulate the function and substrate specificity of mTORC1 and mTORC2. These include mSIN1 and Protor, which seem to preferentially associate within mTORC2, and DEPTOR, which can be found in both mTORCs.

Specific isoforms of many of the mTORC1 and mTORC2 components have also been described and it has been proposed that isoform specific mTORCs may assemble

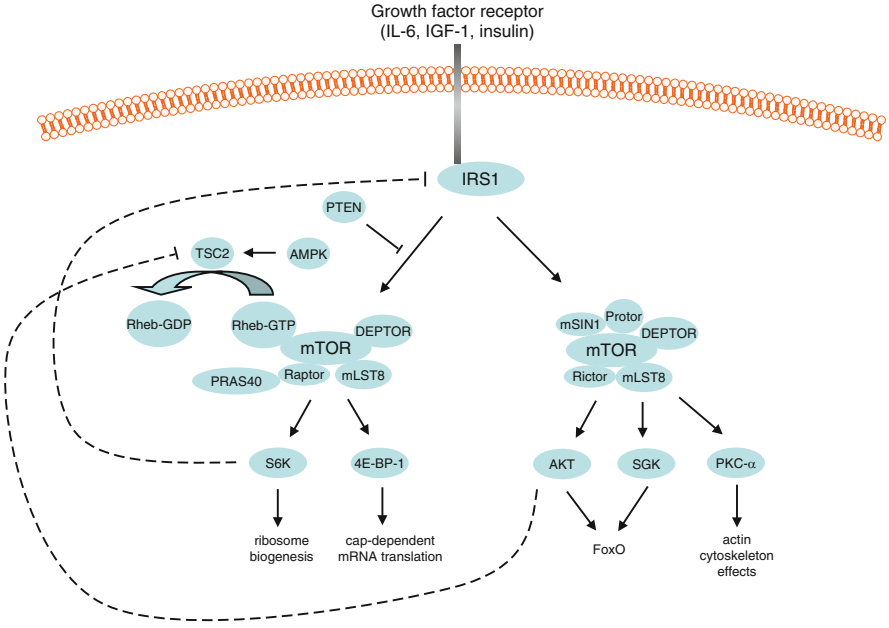


Fig. 6.2 Regulation of mTORC signaling by growth factors. mTORC1 and mTORC2 signaling are activated in response to growth factors including IL-6. mTORC1 signaling is regulated by the tuberous sclerosis complex (TSC1/2), which acts to inhibit mTOR by acting as the GTPase-activating protein (GAP) for Rheb-GTP, which is required for mTORC1 function. AKT activity is induced by growth factor stimulation and mTORC2 and AKT can induce mTOR by inhibiting the function of TSC1/2 and inactivating the mTORC1 negative regulator PRAS40. mTORC1 also regulates the relative flux of growth factor mediated signals via a feedback pathway (*dashed line*) in which S6K phosphorylates IRS1 resulting in its degradation and attenuation of the signaling cascade

in a cell type specific manner leading to varied mTORC1/2 sensitivity to stimuli [24]. The mTORβ isoform displays increased signaling to p70S6K, 4E-BP-1, and AKT [8]. Recently, a cell type specific isoform of Raptor has also been described although the functional significance of this isoform remains to be determined [25]. Isoforms of SIN1 and Protor have also been described which are capable of forming complexes within mTORC2 [24, 26]. Additionally, Hsp70 has been demonstrated to interact with rictor and promote the assembly and kinase activity of mTORC2 following heat shock [27].

6.4 Regulation of mTOR Activation

Significant progress has been made in understanding how various environmental signals regulate mTORC1 activity, while those that regulate mTORC2 are largely unknown (see Fig. 6.2). Growth factor-dependent signaling to mTORC1 is positively stimulated by the GTP-binding protein Rheb, which is negatively regulated by the action of the tuberous sclerosis tumor suppressor proteins TSC1/TSC2 [28]. TSC2 is a GTPase-activating protein and the loss or mutation of TSC2 results in the constitutive activation of Rheb and consequently activation of mTORC1. The TSC1/2 complex is believed to function as a nutrient responsive integrator of signaling to mTORC1. Cell signaling as a result of global cellular energy loss either by AMPK, MAPK, hypoxia, or WNT signaling feed into the TSC1/2/Rheb cascade to regulate mTORC1. AMPK is also known to directly phosphorylate Raptor and disrupt its interaction with mTOR, thereby blunting mTORC1 activity [29].

AKT and mTORC1 mutually regulate the activity of each other in a complex cross-talk. AKT acts in several distinct pathways to regulate mTORC1 activity. First, it can directly phosphorylate TSC2 leading to dissociation of the TSC1/2 complex resulting in activation of mTORC1 as described above (see Fig. 6.2). Secondly, it can also activate mTORC1 via an inhibitory phosphorylation of the mTORC1-negative regulator PRAS40 [30]. Third, activated AKT stabilizes the surface expression of nutrient and amino acid transporters such as Glut1, which results in increased uptake of nutrients and activation of mTORC1 [31]. MTOR, in turn, can activate AKT (see below) through TORC2-induced phosphorylation of AKT or when mTOR inhibition induces derepression of a negative feedback cascade mediated by p70SK on IRS-1, which results in increased signaling through PI3-K to AKT.

While little is known regarding the environmental signals which regulate mTORC2, recent studies have shed light on at least two signaling inputs to this complex. Clearly, growth factors are able to stimulate mTORC2 as immunoprecipitated mTORC2 from insulin stimulated cells have upregulated in vitro activity as determined by serine 473 phosphorylated AKT [24]. Additionally, mTORC2 has been recently shown to be activated by amino acids [32].

6.5 Regulation of mRNA Translation by mTOR

Upregulated protein translation is part of the malignant phenotype [33] and, as such, becomes an attractive therapeutic target in general. The efficacy of mTOR inhibitors in cancer therapy may be primarily due to their ability to curtail translation so that the regulation of this process may determine relative sensitivity to these drugs. Alterations in a cell's extracellular environment rapidly alter gene expression at the translation level. Subsequent to transcription, an mRNA molecule is translated into protein via the action of the ribosome and this process is delineated by three major points of regulation. These are translation initiation, elongation, and termination. While all three of these steps can be regulated in the cell, the major point of regulation occurs at initiation. In eukaryotes, the majority of all mRNA translation occurs by cap-dependent initiation, wherein specific translation initiation factors interact with the mRNA cap structure to form a productive pre-initiation complex. Alternatively, some mRNAs are able to initiate translation via cap-independent mechanisms through the action of an internal ribosome entry site (IRES) where the ribosome binds to the mRNA leader independent of the cap and initiates protein synthesis. While IRES-mediated mRNA translation initiation is also regulated, the mTORC1 pathway primarily regulates cap-dependent mechanisms. As described earlier, mTORC1 directly phosphorylates the mRNA translational regulators S6K and 4E-BP-1. These downstream effectors have been demonstrated to control ribosome biogenesis, mRNA translation, and cell growth [34].

Although eukaryotic translation initiation factors (eIFs) and ribosomal proteins contain identified phosphorylation sites, the precise function of many of these phosphorylations sites remains unknown [35–38]. It has been proposed that many of the kinases involved in these post-translational phosphorylation events must be in relatively close proximity to their appropriate targets in order for phosphorylation to take place within the preinitiation complex. Recent studies have demonstrated that the eIF3 protein complex acts as a molecular conduit linking mTORC1, 4E-BP-1, and S6K phosphorylation to the ordered assembly of the translation preinitiation complex [39]. Under basal conditions inactive S6K is complexed with eIF3. Following stimulation by growth factors, mTORC1 binds the eIF3–S6K complex, dissociating S6K from the complex and the remaining mTORC1–eIF3 complex becomes associated with the 5′ mRNA cap structure. This interaction correlates with the phosphorylation of S6K at its hydrophobic motif (Thr389).

Cap-dependent protein synthesis is inhibited in the cell when the hypophosphorylated form of 4E-BP-1 binds to the cap binding protein, eIF-4E and competitively inhibits the interactions of the scaffolding protein eIF-4G with eIF-4E [40]. Cell stimulation results in mTORC1 directly phosphorylating 4E-BP-1, thus liberating it from eIF-4E and allowing eIF-4E to interact with eIF-4G and the cap-structure on mRNAs and initiate protein synthesis. The phosphorylation of 4E-BP-1 occurs at multiple residues in an ordered manner. The Thr37 and Thr47 phosphorylations prime 4E-BP-1 for subsequent phosphorylations at Ser65 and Thr70. Inactivation of 4E-BP-1 via substitution mutations of these phosphosites, or treatment with rapamycin significantly reduces cap-dependent translation, but does

not completely abolish protein synthesis. This suggests some redundancy in the mRNA translation signaling pathways [41].

As protein translation may be the ultimate target of mTOR inhibitors, it follows that additional regulation mechanisms that can impact translation may serve as resistance pathways. For example, upregulated IRES-dependent translation of D-cyclin and/or c-myc can rescue tumor cells from rapamycin-induced cytostasis [42]. Additional resistance mechanisms potentially include alterations in the 4EBP-1:eIF4E ratio. For example, resistance to rapamycin is associated with decreased levels of 4EBP-1 [43]. Furthermore, deregulated elevations of eIF4E are found in many malignancies [43] and this could also promote translation and serve as a mechanism of resistance.

6.6 Role of mTOR in Cell Proliferation, Apoptosis, and Angiogenesis in Cancer

The concerted effect of mTOR also plays a major role in the regulation of many tumor cell properties. In mammalian cells, mTORC1 regulates the progression of cells from G1 into the S-phase of the cell cycle by several mechanisms or specific "checkpoints." mTORC1 is known to regulate the levels and activity of many cyclins and cyclin-dependent kinases. mTOR, as a central regulator of cell growth, also regulates cell death in normal and tumor cells. Neoangiogenesis often occurs as a response to intra-tumoral hypoxia leading to the upregulation of hypoxia-dependent gene expression. mTORC1 plays a major role in the regulation of neoangiogenesis in tumor cells. Below we describe how mTOR regulates each of these tumor cell properties.

The CDK/cyclin complexes required for S-phase progression can be inhibited by specific CDK-inhibitor proteins (CDKIs) [44], including p21 (also WAF1 or CIP1) and p27 (Kip1). The expression of both p21 and p27 is regulated at the transcriptional and post-transcriptional levels. Activated CDKs (cyclin D/CDK4,6 and cyclin E/CDK2) can phosphorylate Rb, thus removing its inhibitory effect on the E2F family of transcription factors. These transcription factors upregulate the transcription of genes required for S-phase cell cycle progression. The CDKI, p27Kip1, inhibits both cyclin D/CDK4 as well as cyclin E/CDK2 complexes and p27 expression is regulated by signaling through mTORC1. P27 protein levels are relatively elevated in resting cells; however, its levels rapidly fall following cell stimulation. Rapamycin can inhibit growth factor mediated reductions in p27 protein levels in most tumor cell types [45]. Additionally, ectopic expression of a constitutively active form of 4E-BP-1 can enhance the expression of p27 [46].

As a viability-promoting pathway, the mTORC1/S6K axis serves an important function in delivering survival signals by S6K-mediated phosphorylation of the proapoptotic effector BAD [47, 48]. S6K has been shown to directly phosphorylate BAD on Ser136 both in vitro and in vivo and thereby inactivate its proapoptotic

function. Conversely, inhibition of mTORC1/S6K signaling via rapamycin or S6K gene disruption prevents growth factor induced phosphorylation of BAD. In addition, mTOR provides an anti-apoptotic function by upregulating protein phosphatase 5 activity which inhibits apoptosis signal-regulating kinase 1 (ASK 1) and downregulates the apoptosis-inducing stress activated protein kinase pathway [49]. Finally, the mTOR-facilitated expression of anti-apoptotic proteins like MCL-1 [50] can be crucial in maintaining viability of certain tumor types.

In terms of mTORC1 regulation of neoangiogenesis, hypoxia driven upregulation of the transcription factor HIF-1a has been shown to be mTORC1-dependent [51]. The upregulation of HIF-1a results in activation of VEGF its target gene. These observations have positioned mTORC1 as an upstream activator of HIF-1alpha in cancer cells and furthermore suggested that the antitumor activity of rapamycin is mediated, in part, through the inhibition of hypoxia induced stress.

6.7 Activation of mTOR in Multiple Myeloma

Early studies [52–55] identified activation of the PI3-K/AKT pathway in MM cell lines as well as primary cells, which resulted in proliferative and anti-apoptosis responses. It was, thus, natural to investigate which signal proteins downstream of PI3-K/AKT were critical for these responses. A previous study by Chauhan et al. [56] had demonstrated that the IL-6 myeloma growth factor promoted p70S6kinase activity in MM cells. As p70 is a proximal substrate of TORC1 (see above) this suggested activation of mTOR was a downstream target of PI3-K/AKT in myeloma. This notion was confirmed in subsequent studies by Shi et al. [57, 58] where IL-6 or IGF-1 exposure upregulated phosphorylation of the mTOR substrates p70S6K and 4E-BP1 in a PI3-K/AKT-dependent fashion. In addition, prevention of p70 activation and 4E-BP1 phosphorylation by the mTOR inhibitors rapamycin and temsirolimus resulted in inhibition of cytokine-induced MM cell growth [57, 58]. These studies collectively confirmed hyperactivation of mTOR in myeloma downstream of PI3-K/AKT. However, it is not completely clear whether all pro-myeloma growth signals from PI3-K/AKT are mediated by mTOR. Against this hypothesis is the fact that inhibiting PI3-K/AKT often induces MM cell apoptosis while inhibiting TORC1 with first generation mTOR inhibitors only induces G1 arrest without MM cell death. In addition, there are many AKT substrates that are critical for tumor cell health that are independent of mTOR. On the other hand, it is now clear that a more extensive inhibition of TORC1 with more potent second generation TOR inhibitors or the addition of TORC2 inhibition can induce MM cell death (see below). This important question speaks to the potential of targeting the translation apparatus in myeloma.

There are several potential mechanisms by which mTOR might be activated in MM. The study by Guglielmelli et al. [59] suggests that activation correlates with

AKT activation and is, thus, usually downstream of PI3-K/AKT in MM specimens. In addition to the stimulating effects of IL-6 and/or IGF-1 described above, loss-of-function mutations of the tumor suppressor PTEN gene in several MM cell lines results in hyperphosphorylation of mTOR substrates [58] which can be reversed by transfection-induced reexpression of PTEN. Although genetic alterations of PTEN have only been described in relatively few patients [60], epigenetic mechanisms may occur and there are no studies that have assayed PTEN protein expression in MM specimens. In contrast to PTEN, gain-of-function N-RAS or K-RAS mutations are relatively common in MM and correlate with aggressive phenotype. Over-expression of mutant K-RAS or N-RAS in the ANBL-6 MM cell line results in upregulated, rapamycin-sensitive p70 phosphorylation [61] and mutant N-RAS in the same cell line results in upregulation of AKT S473 phosphorylation [62]. These data suggest that MM RAS mutations could activate TORC1 (phosphorylating kinase for p70) and TORC2 (phosphorylating kinase of AKT-S473). However, supportive data on primary specimens linking RAS mutation to mTOR activation is lacking.

6.8 Activity of mTOR in MM

A host of responses occur in MM cells downstream of mTOR activation. The immediate TORC1 substrates p70 and 4E-BP1 are known to be hyperphosphorylated [57, 58] and 4E-BP1 phosphorylation is associated with inhibited binding to the eIF-4E translation initiation factor [58]. Release of eIF-4E allows it to participate in translation initiation complexes with resulting cap-dependent translation. As for TORC2, phosphorylation of the TORC2 substrate AKT S473 is easily detected by IHC in primary samples [62, 63]. The AKT S473 phosphorylation is relatively specific for MM tumor cells as adjacent nonmalignant hematopoietic cells in patient marrows are usually negative for S473 staining. MM cell line studies suggest an additional TORC2 target, SGK-1, is also upregulated as its substrate, NDRG1, is hyperphosphorylated [62].

As p70 and 4E-BP1 phosphorylation promote cap-dependent translation of critical proliferative, survival and angiogenic proteins, it is not surprising that mTOR activation results in upregulated D-cyclin, c-myc and VEGF translation and expression [58, 64, 65]. D-Cyclin and c-myc translation facilitate G1-to-S cell cycle transit and VEGF translation promoted angiogenesis in MM xenografts [64]. Activation of mTOR also suppresses autophagy in MM cell lines [66]. It is also clear that either MM TORC1 and/or TORC2 activity promote MM cell viability [62, 67, 68] although the mechanism of such a tumor-promoting function is not yet clear.

6.9 Preclinical Activity If TORC1 Inhibitors

Rapamycin and the rapalog temsirolimus are effective cytoreductive agents in vitro against MM cell lines [57, 58, 61]. The major effect of these agents is to induce G1 arrest which correlates with their ability to downregulate expression of D-cyclins and c-myc and upregulate p27 [58, 68]. The IC_{50} doses of these drugs against sensitive MM clones are very low (~1–10 nM) [58, 69]. However, the dose response curves are flat, reaching early plateaus in efficacy at only 40–60% inhibition of most MM cell lines [62]. In addition, rapamycin-induced cytotoxicity is significantly reduced by exposure to IL-6, IGF-1 and the presence of bone marrow stromal cells (BMSCs) [68, 69]. In contrast to these results with cell lines, the in vitro effect of rapamycin against primary specimens includes apoptosis in addition to G1 arrest [68]. It is unclear why primary cells would be more sensitive to apoptosis induction and this issue merits further study. Several possibilities come to mind such as: (1) Treated primary MM cells may demonstrate a more profound inhibition of protein translation or a greater sensitivity to protein translation inhibition; (2) Primary cells may exhibit less feedback activation of PI3K/AKT (see below); (3) Additional mTOR-dependent but translation-independent pro-apoptotic effects may singularly occur in primary cells. It should also be noted that apoptosis can be induced in cell lines of other tumor models when exposed to first generation rapalogs [49] depending on the particular genetic makeup of those cells.

A fair amount of literature [70, 71] indicates that tumor cells with elevated AKT activity as a result of dysregulated PI3K activity, AKT gene amplification or loss of PTEN, display markedly increased G1 arrest following rapamycin exposure relative to cells with quiescent AKT. This is also true for MM clones with hypersensitivity to temsirolimus present in AKT-activated PTEN-null MM lines [58], AKT-activated, RAS-mutated lines [61] and AKT-transfected U266 cells [65]. Since heightened AKT activity is a mechanism of resistance to most anti-tumor agents, its sensitizing effect to rapalogs may have clinical relevance and suggest baseline AKT activity as a positive biomarker for future clinical trials of rapalogs. The mechanism by which AKT regulates sensitivity appears explained by a greater rapalog-induced downregulation of D-cyclin translation in “high-AKT” clones [65]. This is due to AKT’s inhibitory effect on the fail-safe cap-independent pathway of protein translation when mTOR-mediated cap-dependent translation is prevented [65]. Whether this sensitizing effect of AKT to first generation rapalogs will be similarly present when second generation TOR inhibitor (see below) are investigated in future studies remains to be seen. As these inhibitors also curtail AKT activity (due to TORC2 inhibition) it may be counter-intuitive to expect that heightened basal AKT activity would sensitize to these agents. However, if their greater efficacy is mostly due to a more profound inhibition of cap-dependent translation, the inhibitory effect of AKT on cap-independent translation may allow greater downregulation of critical proteins involved in cell cycle transit and viability. In addition, heightened AKT activity may suggest AKT addiction as shown with direct AKT inhibitors [63], also resulting in sensitivity to TORC2 inhibition as seen with second generation TOR inhibitors.

It is clear from several studies that when rapalogs are combined with a diverse assortment of anti-MM agents, synergistic cytotoxicity ensues. As most molecularly targeted agents show minimal efficacy in clinical trials as single agents, these preclinical studies are important in suggesting which combinations may be efficacious in patients. Synergistic anti-MM activity has been most notable when first generation mTOR inhibitors are combined with dexamethasone [67, 68], Revlimid [69] and the 17-AAG HSP90 inhibitor [72]. Importantly, the apoptotic response to combination therapy was markedly enhanced in these studies and the protective effect of IL-6, IGF-1 and BMSCs was overcome [68, 69]. In contrast, combination of rapamycin with bortezomib resulted in antagonistic anti-MM effects [73] quite possibly due to the upregulation of AKT activity observed secondary to mTOR inhibition (below). Since the major molecular effect of rapamycin is to curtail cap-dependent translation, it is possible that translation inhibition sensitizes to apoptotic effects of different myeloma therapies. In fact, inhibition of cap-dependent translation in MM cells by gene transfer also sensitizes to dexamethasone-induced apoptosis [67]. A high throughput screen for mRNA translational state identified several anti-apoptotic proteins whose translation was inhibited by rapamycin [67] suggesting an explanation for the sensitizing effect of rapalogs.

Rapalogs have also demonstrated anti-MM effects in murine models. Temsirolimus [74] and nanoparticle albumin-bound rapamycin [75] have shown such in vivo efficacy, the latter especially when combined with perifosine. Effective anti-MM doses of these agents produced little host toxicity and were associated with decreased tumor cell proliferation, induction of apoptosis and prevention of neo-angiogenesis. Additionally, cytoreduced tumors exhibited reduced c-myc, D-cyclin and VEGF expression [64, 65, 74] and elevated p27 expression [74], results which mirrored that from in vitro exposure of cell lines to rapalogs. It is also noteworthy that the in vitro findings of AKT activity sensitizing MM cells to rapalogs and synergistic interaction between rapalogs and dexamethasone were reproduced in these in vivo models [67, 74]. The one notable difference between in vitro and in vivo studies was the significant induction of tumor cell apoptosis in vivo which is lacking during in vitro exposure to single agent rapalogs. One obvious possible explanation is the requirement for angiogenesis to maintain tumor cell viability in vivo which was significantly prevented by temsirolimus injections in the SC-challenged murine model [74].

6.10 Use of Second-Generation mTOR Inhibitors

It is now apparent that feedback activation of the PI3K/AKT cascade subsequent to mTOR inhibition can dampen antitumor effects. This resistance mechanism exists because the insulin receptor substrate-1 (IRS-1) is a downstream target of mTOR with mTOR or p70-mediated phosphorylation of IRS-1 resulting in dissociation of this adaptor from the IGF-1 receptor and its proteasomal degradation [76]. Both of these events result in downregulation of insulin or IGF-1 signaling through PI3-K to

AKT. Thus, when mTOR/p70-mediated phosphorylation of IRS-1 is prevented by rapalogs, the IGF-R/IRS-1/PI3-K/AKT pathway becomes de-repressed and subsequent AKT activation can prevent tumor cell apoptosis. A study by Shi et al. [73] confirmed the existence of the feedback cascade in MM cells. Exposure of cell lines and primary MM cells to rapamycin resulted in decreased phosphorylation of IRS-1 on serine 312, increased tyrosine phosphorylation of the p85 regulatory subunit of PI3-K, increased PI3k lipid kinase activity, and increased AKT phosphorylation kinase activity. Rapamycin-induced activation of the pathway was prevented by a blocking anti-IGF-R antibody, indicating the importance of proximal IGF-R/IRS-1 signaling. Importantly, the presence of rapamycin and upregulated AKT activity seemed to protect MM cells against bortezomib-induced apoptosis. These results have been confirmed by Cirstea et al. [75] and provide a caveat for arbitrary decisions on combining anti-MM agents with first generation mTOR inhibitors. Nevertheless, these data provide a rationale for combining mTOR and PI3-K/AKT inhibition and support the development of drugs such as NVP-BEZ235, a dual PI3-K/mTOR inhibitor which has shown significant preclinical anti-MM activity [77]. In addition, once it became apparent that mTOR complexed with rictor (TORC2) was the key kinase that phosphorylates AKT on S473 [78], which is required for full AKT kinase activity, the development of drugs that could inhibit TORC2 as well as TORC1 became a priority. There are now several of these compounds available and some are already in clinical phase I trials. In contrast to the first generation rapalogs that bind mTOR adjacent to the kinase domain with allostearic inhibition, these second generation compounds are ATP-competitive inhibitors that directly bind the mTOR catalytic site and, thus, curtail TORC2 as well as TORC1. In MM cell lines and primary specimens, these TORC1/TORC2 inhibitors are more effective than rapamycin on a molar basis for inhibiting cell growth and inducing apoptosis [62, 79, 80] and they markedly synergize with bortezomib [62] while, as described above, rapamycin is antagonistic with bortezomib. These more impressive anti-MM effects are associated with abrogation of TORC2 activity (i.e., prevention of mTOR phosphorylation on S2481, AKT phosphorylation of S473 and NDRG1 phosphorylation on serine 330 (NDRG1 is a substrate of the SGK kinase, the latter which is activated by TORC2 [81]).

Although these initial studies are exciting, there are several questions concerning these newer TORC1/TORC2 inhibitors that need to be addressed: First, it is not clear whether their more impressive activity compared to rapamycin is due to the additional TORC2 inhibition or a more intense TORC1 inhibition. Studies in other tumor models [50, 82] demonstrate that TORC1-induced phosphorylation of 4E-BP1 and liberation of eIF-4E is a relatively rapamycin-resistant activity of TORC1 and that newer inhibitors directly targeting the TOR kinase domain are much more effective in 4E-BP1 dephosphorylation and sequestering of eIF-4E. In MM cell lines, rapamycin can induce 4E-BP1 dephosphorylation and eIF-4E binding although a newer TORC1/TORC2 inhibitor is modestly more effective [62]. On the other hand, knock down of rictor with attendant TORC2 inhibition is deleterious to MM cells [62] indicating that TORC2 is a legitimate target. The answer to whether greater TORC1 inhibition or additional TORC2 inhibition is key to anti-MM

responses will have a great influence on the development of future third generation mTOR inhibitors.

A second important issue is whether these newer agents completely resolve the problem of resistance due to feedback signaling. Although they prevent AKT S473 phosphorylation, feedback activation of IGF-R/PI3-K signaling (due to TORC1/p70 inhibition) may still upregulate AKT T308 phosphorylation which can, in some instances, maintain AKT kinase activity to some degree in the absence of S473 phosphorylation [4]. Amore problematic feedback cascade may be the ERK MAPK pathway. ERK becomes hyperphosphorylated and activated subsequent to treatment with TORC1/TORC2 inhibitors [62] and ERK activity is well known as a pro-growth factor for MM cells [83]. This potential resistance mechanism could be overcome by combining newer mTOR inhibitors with ERK inhibitors, or, alternatively, if TORC2 inhibition is shown to be critical although not involved in ERK feedback activation, developing newer agents that only target TORC2 without effects on TORC1, thus preventing feedback activation of signaling.

6.11 DEPTOR

A recent development that may be relevant to future mTOR targeted therapy in MM is the identification of DEPTOR as an mTOR binding protein that negatively regulates TORC1 and TORC2 activity [84]. Loss of DEPTOR activates TORC1 output (S6K1 activity) and TORC2 output (AKT and SGK1 activity) and, in non-myeloma cells, promotes cell growth and survival. In contrast, ectopic DEPTOR over-expression suppresses S6K activity (i.e., suppresses TORC1) but, via de-repression of feedback regulation of PI3-K signaling, it activates AKT. In an intricate circuit, DEPTOR expression is itself negatively regulated by TORC1 and TORC2. Since the mTOR pathway is hyperactivated in many different tumor models, it is not surprising that expression of this TORC-inhibitory protein is kept at very low levels in malignantly transformed cells. However, in a significant proportion of myeloma samples, DEPTOR is markedly over-expressed, suggesting that it provides a proliferative/survival advantage to MM cells. DEPTOR over-expression was specifically detected in specimens with Ig gene translocations and, most markedly, where C-MAF or MAFB transcription factors were involved and dysregulated in the translocations. Additional experiments identified DEPTOR as a transcriptional target of MAF, explaining the high levels of DEPTOR in MAF-translocated specimens. In MM cell lines with high DEPTOR expression, there was an association with inhibited S6K phosphorylation and elevated AKT phosphorylation. This was explained as the anticipated DEPTOR-induced TORC1 inhibition (S6K output) and the de-repressed PI3K/AKT pathway (increased AKT). The most remarkable aspect of DEPTOR in MM comes from DEPTOR knockdown in these MM cell lines. The TORC1 output is increased as expected (increased S6K phosphorylation) and feedback PI3K/AKT activity is decreased as expected. However, in contrast to non-myeloma cell models where DEPTOR knockdown promoted cell growth, in MM

lines with high DEPTOR expression, knock down resulted in apoptosis. The data suggest that the feedback PI3K/AKT activation in DEPTOR over-expressed MM specimens is a critical survival pathway for these cells. Another possibility relates to the exceedingly high level of protein synthesis in MM cells due to their mandatory Ig secretion. The prime raison d'etre of DEPTOR in those MM cells may be to restrict protein synthesis because they are always primed for death due to hyperactivation of the unfolded protein response (UPR) cascade from excessive ER stress. Markedly turning up the rheostat of the translational machinery secondary to TORC1 upregulation in DEPTOR knocked down cells may tip the scales towards cell death from UPR signaling. Thus, DEPTOR may be a legitimate target for future therapy in a subset of MM patients.

6.12 Clinical Trials in MM Patients

The earliest clinical trials with mTOR inhibitors were performed with the intravenous Wyeth formulation of temsirolimus, an ester of rapamycin. The drug was generally well tolerated and peak plasma concentrations were above that required for mTOR inhibition. An intermittent weekly regimen has been established although a lower dose daily schedule might theoretically permit a more consistent mTOR inhibition in tumor cells [85]. A second orally administered mTOR inhibitor, everolimus, can be given daily and may achieve this goal. The phase I trials suggested some efficacy in renal cell CA which has been subsequently confirmed and these mTOR inhibitors are now part of the armamentarium used in this disease [86, 87]. Efficacy has also been demonstrated in mantle cell lymphoma patients [88] and approval has followed for this disease as well.

One clinical trial of temsirolimus as a single agent in relapsed/refractory MM patients has been completed and reported [89]. Sixteen heavily pretreated patients were treated with a relatively low dose of temsirolimus (25 mg IV q week as opposed to the starting dose of 250 mg IV q week used in MCL patients). However, even at this starting dose, further dose reductions occurred for AEs in 11 of 16 patients although there were no treatment-related deaths. Toxicity was mostly hematologic. Efficacy was very minimal with one partial response and five minor responses. There were rough correlations between responsiveness and the temsirolimus AUC as well as with the pharmacodynamic effect of drug assessed by immunoblot assay for phosphorylation of p70S6K and phosphorylation of 4E-BP1 in circulating PBMNCs. Guenther et al. [90] has shown similar results using oral everolimus. Somewhat less toxicity was seen and one PR and four stable diseases were identified in seven evaluable patients.

In contrast to the above studies in MM patients, a more marked antitumor effect has been seen in two related disease, mantle cell lymphoma (MCL) and Waldenstrom's macroglobulinemia. At a starting dose of 250 mg IV q week, temsirolimus resulted in a 38% overall response rate in patients with MCL with 1 CR and 12 PRs in 34 evaluable patients [88]. The major molecular similarity to MM in MCL is the

D-cyclin translocation and dysregulated expression. As described above, D-cyclin translation is a major target of mTOR inhibitors and cell line responsiveness to mTOR inhibitors correlates well with the degree of D-cyclin downregulated expression. Obviously, there are many other differences in the molecular pathology between MCL and MM that may relate to differential responsiveness. Although the starting dose in the MCL study was considerably higher than in the MM studies, significant dose reductions occurred in >90% of MCL patients with a median monthly dose of 525 mg. Furthermore, there was no significant difference in monthly dose seen between responders and non-responders.

Waldenstrom's disease is another more responsive B cell malignancy with a high single agent activity signal [91]. In 50 evaluable heavily pretreated patients (median of 3 prior therapies), receiving everolimus at a starting dose of 10 mg PO qd, 42% achieved a PR and 28% additional patients achieved minimal response (MR). Stable disease was seen in 16%. Responses were associated with correction of anemia and decreases in lymphadenopathy. The fact that Waldenstrom tumor cells appear to have lost PTEN RNA and protein expression [92] may explain this greater efficacy. This would be consistent with preclinical observations that tumors with genetic abnormalities affecting pathways that regulate mTOR are, in fact, much more dependent on mTOR function. This is true for MM as well (see above [58]) and suggest that the relatively small numbers of MM patients with PTEN loss could be more responsive to mTOR inhibitors.

Because of the modest efficacy of mTOR inhibitors when used alone, several trials have been designed to combine them with other known anti-MM drugs. In a phase I/II study combining bortezomib with temsirolimus [93], an MTD was identified as 1.6 mg/m^2 bortezomib q week and 25 mg temsirolimus q week and toxicity was generally manageable (mostly cytopenias). In the phase II part of the trial an overall response rate of 47% was seen in 43 patients with 5% CR, 9% VGPR, 19% PR and 14% MR. Of the patients considered refractory to prior bortezomib, the response rate was considerably lower at 21% although there were still 3 PRs and 3 MRs in 32 patients suggesting activity of temsirolimus.

Because of preclinical evidence of synergy against MM cells when mTOR inhibitors are combined with Revlimid [69], this combination has also been tested in clinical trials. The Boston group [94] combined everolimus with Revlimid and, in 19 evaluable patients, there were 1 CR, 2 PRs and 8 MRs. It was not clear, however, what the contribution of the mTOR inhibitor was to these responses as only three patients in the trial were thought to be refractory to Revlimid. The Ohio State group [95] combined Revlimid with temsirolimus and demonstrated a similar low level of response rate with 1 CR, 1 PR and 3 MR in 21 patients.

It is difficult to make any clear conclusions from the above phase I/II trials that have studied small numbers of patients. The first generation mTOR inhibitors (temsirolimus and everolimus) have been generally well tolerated although toxicity is increased when combined with other agents [95]. As mTOR inhibitors are well known immune-suppressive drugs, it is gratifying to note there have not been serious infectious complications in these early trials even though the patients have been heavily pretreated. The issues of dose, scheduling and potential predictive biomarkers

have also not been clearly defined. More importantly, it is likely that these questions will never be answered for rapalog use in MM as the field rapidly moves forward to focus on the second generation TORC1/TORC2 inhibitors. These are already in trials and results are eagerly awaited.

References

1. Vezina C, Kudelski A, Sehgal SN (1975) Rapamycin, a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. J Antibiot 28:721–726
2. Heitman J, Movva N, Hall H (1991) Target for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science 253:905–909
3. Alessi D, Pearce L, Garcia-Martinez J (2009) New insight into mTOR signaling: TORC2 and beyond. Sci Signal 2:1–4
4. Guertin D, Sabatini D (2009) The pharmacology of mTOR inhibitors. Sci Signal 2:1–6
5. Andrade MA, Petosa C, O'Donoghue S et al (2001) Comparison of ARM and HEAT protein repeats. J Mol Biol 309:1–18
6. Kim D, Sarbassov D, Ali S et al (2002) mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 110:163–175
7. Liu X, Zheng XF (2007) Endoplasmic reticulum and Golgi localization sequences for mammalian target of rapamycin. Mol Biol Cell 18:1073–1082
8. Panasyuk G, Nemazanny I, Zhyvoloup A et al (2009) The mTORbeta splicing isoform promotes cell proliferation and tumorigenesis. J Biol Chem 284:30807–30814
9. Perry J, Kleckner N (2003) The ATRs, ATMs and TORs are giant HEAT repeat proteins. Cell 112:151–155
10. Dames SA, Mulet J, Rathgeb-Skabo K et al (2005) The solution structure of the FATC domain of the protein kinase target of rapamycin suggests a role for redox-dependent structural and cellular stability. J Biol Chem 280:20558–20564
11. Choi J, Chen J, Schreiber S et al (1996) Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. Science 273:239–242
12. Long X, Lin Y, Ortiz-Vega S et al (2005) Rheb binds and regulates the mTOR kinase. Curr Biol 15:702–713
13. Sturgill T, Hall M (2009) Activating mutations in TOR in similar structures as the oncogenic mutations in PI3KCalpha. ACS Chem Biol 4(12):999–1015
14. Holz M, Blenis J (2005) Identification of S6 kinase 1 as a novel mammalian target of rapamycin (mTOR)-phosphorylating kinase. J Biol Chem 280:26089–26093
15. Chiang G, Abraham R (2005) Phosphorylation of mammalian target of rapamycin (mTOR) at Ser-2448 is mediated by p70S6 kinase. J Biol Chem 280:25485–25490
16. Peterson R, Beal P, Comb M et al (2000) FKBP12-rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions. J Biol Chem 275:7416–7423
17. Edinger A, Thompson C (2004) An activated mTOR mutant supports growth factor-independent, nutrient-dependent cell survival. Oncogene 23:5654–5663
18. Banaszynski L, Liu C, Wandless T (2005) Characterization of the FKBP.rapamycin.FRB ternary complex. J Am Chem Soc 127:4715–4721
19. Hara K, Maruki Y, Long X et al (2002) Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell 110:177–189
20. Sarbassov D, Ali S, Kim D et al (2004) Rictor, a novel partnet of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol 14:1296–1302
21. Jacinto E, Loewith R, Schmidt A et al (2004) Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol 6:1122–1128

22. Sarbassov D, Guertin D, Ali S et al (2005) Phosporylation and regulation of AKT/PKB by the rictor-mTOR complex. Science 307:1098–1101
23. Garcia-Martinez J, Alessi D (2008) mTOR complex 2 (mTORC 2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). Biochem J 416:375–385
24. Frias M, Thoreen C, Jaffe J et al (2006) mSIN1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. Curr Biol 16:1865–1870
25. Sun C, Southard C, Di Rienzo A et al (2009) Characterization of a novel splicing variant in the RAPTOR gene. Mutat Res 662:88–92
26. Pearce L, Huang X, Boudeau J et al (2007) Identification of Protor as a novel Rictor-binding component of mTOR complex-2. Biochem J 405:513–522
27. Martin J, Masri J, Bernath A et al (2007) Hsp70 associates with Rictor and is required for mTORC2 formation and activity. Biochem Biophys Res Commun 372:578–583
28. Zhang Y, Gao X, Saucedo L et al (2003) Rheb is a direct target of the tuberous sclerosis tumor suppressor proteins. Nat Cell Biol 5:578–581
29. Gwinn D, Shackelford D, Egan D et al (2008) AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell 30:214–226
30. Sancak Y, Thoreen C, Peterson T et al (2007) PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Mol Cell 25:903–915
31. Edinger A, Thompson C (2002) Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. Mol Biol Cell 13:2276–2288
32. Tato I, Bartrons R, Ventura F et al (2010) Amino acids activate mTOR complex 2 via PI3K/AKT signaling. J Biol Chem 286:6128–6142. doi:10.1074/jbc.M110.166991
33. DeBenedetti A, Harris A (1999) eIF4E expression in tumors: its possible role in progression of malignancies. Int J Biochem Cell Biol 31:59–72
34. Hay N, Sonenberg N (2004) Upstream and downstream of mTOR. Genes Dev 18:1926–1945
35. Loeb J, Blat C (1970) Phosphorylation of some rat liver ribosomal protein and its activation by cyclic AMP. FEBS Lett 10:105–108
36. Kabat D (1970) Phosphorylation of ribosomal proteins in rabbit reticulocytes. Characterization and regulatory aspects. Biochemistry 9:4160–4175
37. Trauch J, Mumby M, Traut R (1973) Phosphorylation of ribosomal proteins by substrate-specific protein kinases from rabbit reticulocytes. Proc Natl Acad Sci USA 70:373–376
38. Kaerlein M, Horak I (1976) Phosphorylation of ribosomal proteins in HeLa cells infected with vaccinia virus. Nature 259:150–151
39. Holz M, Ballif B, Gygi S et al (2005) mTOR and S6K mediate assembly of the translation initiation complex through dynamic protein interchange and ordered phosphorylation events. Cell 123:569–580
40. Gingras A, Raught B, Gygi S et al (2001) Hierarchical phosphorylation of the translation inhibitor 4E-BP1. Genes Dev 15:2852–2864
41. Clemens M (2001) Translational regulation in cell stress and apoptosis. Roles of the eIF4E binding proteins. J Cell Mol Med 5:221–239
42. Shi Y, Sharma A, Wu H et al (2005) Cyclin D1 and c-myc IRES-dependent translation is regulated by AKT activity and enhanced by rapamycin through a p38 MAPK and ERK-dependent pathway. J Biol Chem 280:10964–10973
43. Huang S, Bjornsti M, Houghton P (2003) Rapamycins: mechanism of action and cellular resistance. Cancer Biol Ther 2:222–232
44. Sherr C, Roberts J (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 13:1501–1512
45. Luo Y, Marx S, Kiyokawa H et al (1996) Rapamycin resistance tied to defective regulation of p21kip1. Mol Cell Biol 16:6744–6751
46. Jiang H, Coleman J, Miskimins R et al (2003) Expression of constitutively active 4EBP-1 enhances p27Kip1 expression and inhibits proliferation of MCF7 breast cancer cells. Cancer Cell Int 3:2

47. Djouder N, Metzler S, Schmidt A et al (2007) S6K1-mediated disassembly of mitochondrial UR1/PP1gamma complexes activates a negative feedback program that counters S6K1 survival signaling. Mol Cell 28:28–40
48. Harada H, Andersen J, Mann M et al (2001) p70S6 kinase signals call survival as well as growth, inactivating the pro-apoptotic molecule BAD. Proc Natl Acad Sci USA 98:9666–9670
49. Huang S, Shu L, Easton J et al (2004) Inhibition of mTOR activates apoptosis signal-regulating kinase 1 signaling by suppressing protein phosphatase 5 activity. J Biol Chem 279:36490–36496
50. Hsieh AC, Costa M, Zollo O et al (2010) Genetic dissection of the oncogenic mTOR pathway reveals druggable addiction to translational control via 4EBP-eIF4E. Cancer Cell 17:249–261
51. Hudson C, Liu M, Chiang G et al (2002) Regulation of hypoxia-inducible factor 1alpha expression and function by the mammalian target of rapamycin. Mol Cell Biol 22:7004–7014
52. Tu Y, Gardner A, Lichtenstein A (2000) The Phosphatidylinositol 3-kinase/AKT kinase pathway in multiple myeloma plasma cells: roles in cytokine-dependent survival and proliferative responses. Cancer Res 60:6763–6770
53. Hsu J, Shi Y, Krajewski S et al (2001) The AKT kinase is activated in multiple myeloma tumor cells. Blood 98:2853–2855
54. Hyun T, Yam A, Pece S et al (2000) Loss of PTEN expression leading to high AKT activation in human multiple myelomas. Blood 96:3560–3568
55. Ge NL, Rudikoff S (2000) Insulin-like growth factor I is a dual effector of multiple myeloma cell growth. Blood 96:2856–2861
56. Chauhan D, Pandey P, Ogata A et al (1997) Dexamethasone induces apoptosis of multiple myeloma cells in a JNK/SAP kinase independent mechanism. Oncogene 15:837–843
57. Shi Y, Hsu J, Hu L et al (2002) Signal pathways involved in activation of p70S6K and phosphorylation of 4E-BP1 following exposure of multiple myeloma tumor cells to IL-6. J Biol Chem 277:15712–15720
58. Shi Y, Gera J, Hu L et al (2002) Enhanced sensitivity of multiple myeloma cells containing PTEN mutations to CCI-779. Cancer Res 62:5027–5034
59. Guglielmelli T, Cappia S, Giugliano E et al (2008) The AKT/mTOR/P70S6K/4EB-P1 signaling pathway is activated in a subset of multiple myeloma patients and correlates with high serum levels of beta 2 microglobulin. In: Abstracts of the American Society for Hematology, December 2008
60. Chang H, Xy Q, Claudio J et al (2006) Analysis of PTEN deletions and mutations in multiple myeloma. Leuk Res 30:262–265
61. Hu L, Shi Y, Hsu J et al (2003) Downstream effectors of oncogenic ras in multiple myeloma cells. Blood 101:3126–3135
62. Hoang B, Frost P, Shi Y et al (2010) Targeting TORC2 in multiple myeloma with a new mTOR kinase inhibitor. Blood 116:4560–4568
63. Zollinger A, Stuhmer T, Chatterjee M et al (2008) Combined functional and molecular analysis of tumor cell signaling defines 2 distinct myeloma subgroups: AKT-dependent and AKT-independent multiple myeloma. Blood 112:3403–3411
64. Frost P, Shi Y, Hoang B et al (2007) AKT activity regulates the ability of mTOR inhibitors to prevent angiogenesis and VEGF expression in multiple myeloma cells. Oncogene 26:2255–2262
65. Frost P, Shi Y, Hoang B et al (2009) Regulation of D-cyclin translation inhibition in myeloma cells treated with mTOR inhibitors: rationale for combined treatment with ERK kinase inhibitors and rapamycin. Mol Cancer Ther 8:83–93
66. Hoang B, Benavides A, Shi Y et al (2009) Effect of autophagy on multiple myeloma cell viability. Mol Cancer Ther 8:1974–1984
67. Yan H, Frost P, Shi Y et al (2006) Mechanism by which mTOR inhibitors sensitize multiple myeloma cells to dexamethasone-induced apoptosis. Cancer Res 66:2305–2313
68. Stromberg T, Dimberg A, Hammarberg A et al (2004) Rapamycin sensitizes multiple myeloma cells to apoptosis induced by dexamethasone. Blood 103:3138–3147

69. Raje N, Kumar S, Hideshima T et al (2004) Combination of the mTOR inhibitor rapamycin and CC-5013 has synergistic activity in multiple myeloma. Blood 104:4188–4193
70. Neshat MS, Mellinghoff IK, Tran C et al (2001) Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. Proc Natl Acad Sci USA 98:10314–10319
71. Podsypanina K, Lee RT, Politis C et al (2001) An inhibitor of mTOR reduces neoplasia and normalizes p70S6 kinase activity in PTEN +/− mice. Proc Natl Acad Sci USA 98:10320–10325
72. Alsayed F, Leleu X, Jia X et al (2006) Combination mTOR inhibitor rapamycin and HSP90 inhibitor 17 allylamino 17-demethoxygeldanamycin has synergistic activity in multiple myeloma. Clin Cancer Res 12:6826–6835
73. Shi Y, Yan H, Frost P et al (2005) Mammalian target of rapamycin inhibitors activate the AKT kinase in multiple myeloma cells by up-regulating the insulin-like growth factor receptor/insulin receptor substrate-1/phosphatidylinositol 3-kinase cascade. Mol Cancer Ther 4:1533–1540
74. Frost P, Moatamed F, Hoang B et al (2004) In vivo antitumor effects of the mTOR inhibitor CCI-779 against human multiple myeloma cells in a xenograft model. Blood 104:4181–4187
75. Cirstea D, Hideshima T, Rodig S et al (2010) Dual inhibition of AKT/mTOR pathway by nanoparticle albumin-bound-rapamycin and perifosine induces antitumor activity in multiple myeloma. Mol Cancer Ther 9:963–975
76. O'reilly K, Rojo F, She Q et al (2006) MTOR inhibition induces upstream receptor tyrosine kinase signaling and activates AKT. Cancer Res 66:1500–1508
77. McMillin DW, Ooi M, Delmore J et al (2009) Antimyeloma activity of the orally bioavailable dual phosphatidylinositol 3-kinase/mTOR inhibitor NVP-BEZ235. Cancer Res 69:5835–5842
78. Guertin D, Stevens D, Thoreen C et al (2006) Ablation in mice of the mTORC components raptor, rictor or mLST8 revelas that mTORC2 is required for signaling to AKT-FOXO but not S6K1. Dev Cell 11:859–871
79. Cirstea D, Hideshima T, Santo L et al (2010) Disruption of DEPTOR/TORC1/TORC2 signaling cascade using a novel selective mTOR inhibitor AZD8055 results in growth arrest and apoptosis of multiple myeloma cells. Proceed of American Society of Hematology, December 2010
80. Maiso P, Azab A, Liu Y et al (2010) Dual targeting of TORC1 and TORC2 as a new strategy in the treatment of multiple myeloma. Proceed of American Society of Hematology, December 2010
81. Murray J, Campbell D, Morrice N et al (2004) Exploitation of KESTREL to identify NDRG family members as physiological substrates for SGK1 and GSK3. Biochem J 384:477–488
82. Janes MR, Limon JJ, So L et al (2010) Effective and selective targeting of leukemia cells using a TORC1/2 kinase inhibitor. Nat Med 16:205–213
83. Ogata A, Chauhan D, Teoh G et al (1997) IL-6 triggers cell growth via the RAS-dependent MAP kinase cascade. J Immunol 159:2212–2221
84. Peterson TR, Laplante M, Thoreen C et al (2009) DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. Cell 137:1–14
85. Sawyers C (2003) Will mTOR inhibitors make it as cancer drugs? Cancer Cell 4:343–348
86. Hudes G, Carducci M, Tomczak P et al (2007) Temsirolimus, interferon alfa, or both for advanced renal cell carcinoma. N Engl J Med 356:2271–2281
87. Hainsworth J, Spigel D, Burris H et al (2010) Phase II trial of bevacizumab and everolimus in patients with advanced renal cell carcinoma. J Clin Oncol 28:2131–2136
88. Witzig T, Geter S, Ghobrial I et al (2005) Phase II trial of single agent temsirolimus for relapsed mantle cell lymphoma. J Clin Oncol 23:5347–5356
89. Farag SS, Zhang S, Jansak B et al (2009) Phase II trial of temsirolimus in patients with relapsed or refractory multiple myeloma. Leuk Res 33:1475–1480
90. Guenther A, Baumann P, Burger R et al (2009) Phase I/II study with single agent everolimus in patients with relapsed or refractory multiple myeloma. Proceed of American Society for Hematology, December 2009

91. Ghobrial IM, Gertz M, LaPlant B et al (2010) Phase II trial of the oral mTOR inhibitor everolimus in relapsed or refractory Waldenstrom Macroglobulinemia. J Clin Oncol 28:1408–1414
92. Roccaro A, Sacco A, Husu E et al (2010) Dual targeting of the PI3K/AKT/mTOR pathway as an antitumor strategy in Waldenstrom Macroglobulinemia. Blood 115:559–569
93. Ghobrial IM, Weller E, Ravi V et al (2010) Final results of the Phase I/II trial of weekly bortezomib in combination with temsirolimus in relapsed or refractory multiple myeloma specifically in patients refractory to bortezomib. Proceed of the American Society for Hematology, December 2010
94. Mahindra A, Richardson P, Hari P et al (2010) Updated results of a phase I study of RAD001 in combination with lenalidomide in patients with relapsed or refractory multiple myeloma with pharmacodynamic and pharmacokinetic analysis. Proceed of American Society for Hematology, December 2010
95. Hofmeister C, Benson D, Efebera Y et al (2009) Phase I trial of lenalidomide and CCI-779 in patients with relapsed multiple myeloma. Proceed of American Society for Hematology. December 2009

Chapter 7
Jak/STAT Signaling in the Pathogenesis and Treatment of Multiple Myeloma

Erik A. Nelson, Sarah R. Walker, and David A. Frank

Abstract The malignant behavior of multiple myeloma cells is driven by the pattern of gene expression exhibited by these cells, particularly inappropriate expression of genes regulating cell survival and proliferation. Working back from this observation, it was found that the transcription factor STAT3 is activated constitutively in a large proportion of cases. Whereas STAT3 is normally activated rapidly and transiently in response to cytokines, the continual activation of STAT3 in myeloma leads to enhanced expression of genes that underlie the proliferation, spread, and therapeutic resistance seen in this disease. This constitutive activation of STAT3 can be mediated by a number of pathways including autocrine or paracrine loops (particularly involving IL-6), as well as a loss of negative regulators of STAT activation. The finding that STAT3 plays an oncogenic role in the biology of multiple myeloma also provides an opportunity to develop molecular therapies that target this pathway. Although transcription factors like STAT3 have traditionally been viewed as difficult targets for pharmacological therapy, rapid advances are being made in targeting this oncogenic transcription factor. Alone or in combination with other therapeutic modalities, STAT3 inhibitors hold great promise for increased efficacy and decreased toxicity in the treatment of patients with multiple myeloma.

Authors Erik A. Nelson and Sarah R. Walker are contributed equally.

E.A. Nelson • S.R. Walker • D.A. Frank, M.D., Ph.D. (✉)
Department of Medical Oncology, Dana-Farber Cancer Institute,
44 Binney Street, Mayer 522B, Boston, MA 02115, USA

Departments of Medicine, Brigham and Women's Hospital, Harvard Medical School,
Boston, MA, USA
e-mail: david_frank@dfci.harvard.edu

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_7,

7.1 Introduction

While multiple myeloma has generally been viewed as an incurable disease when treated with standard cytotoxic chemotherapy, our increasing understanding of the molecular pathogenesis of this disease has revealed new targets that hold out the promise for greatly improved clinical responses. There are two key requirements to developing targeted therapeutic strategies for myeloma. The first is to elucidate pathways that are activated in a myeloma cell and drive the pathogenesis of the malignancy. The second is that the target must either be unique to the myeloma cell or dispensable in normal cells. Given that the pathogenesis of multiple myeloma reflects processes such as inappropriate survival and proliferation that are directly related to the pattern of gene expression in the cell, one area of particular focus has been transcription factors that are activated inappropriately in a myeloma cell.

7.1.1 STAT3 As an Oncogenic Transcription Factor

Transcription factors were among the earliest oncogenes identified, including *myc*, *fos*, and *jun*. However, it is becoming increasingly apparent that even in the absence of mutation, constitutive activation of a transcription factor can be a key step in the molecular pathogenesis of cancer. These so-called oncogenic transcription factors can serve as convergence points of multiple signaling pathways, and regulate expression of a key cohort of genes that drive the malignant behavior of a myeloma cell [1]. One transcription factor that has emerged as an important oncogenic regulator in myeloma is STAT3. STAT3 is one of seven STAT family members that relay signals emanating from cytokine and growth factor receptors at the cell surface to the nucleus [2]. STAT3, like all STATs, is present in the cytoplasm under resting conditions. When a single tyrosine residue located near the carboxyl terminus of STAT3 becomes phosphorylated, STAT3 forms dimers through reciprocal interactions between the phosphorylated tyrosine of one STAT3 molecule with the Src homology 2 (SH2) domain of its binding partner. Dimer formation then allows the nuclear accumulation of STAT3, where it can then bind to nine base pair sequences in the regulatory region of target genes, thereby modulating gene transcription (Fig. 7.1). While STATs (an acronym of *s*ignal *t*ransducers and *a*ctivators of *t*ranscription) are generally viewed as activators of gene transcription, increasing evidence indicates that STATs may also repress the expression of target genes as well [3]. Although it was initially proposed that STATs are present in the cytoplasm as monomers under basal conditions, more recent evidence suggests that STATs exist as dimers prior to activation but that their conformation is altered by tyrosine phosphorylation, thereby allowing nuclear localization [4]. Even in the absence of phosphorylation, STAT3 may shuttle between the nucleus and the cytoplasm [5]. However, the conformational change induced by tyrosine phosphorylation leads to a masking of nuclear export signals, thereby promoting their nuclear accumulation.

Understanding the molecular steps in the activation of STAT3 has allowed the generation of artificial mutant forms of the protein which have served as important

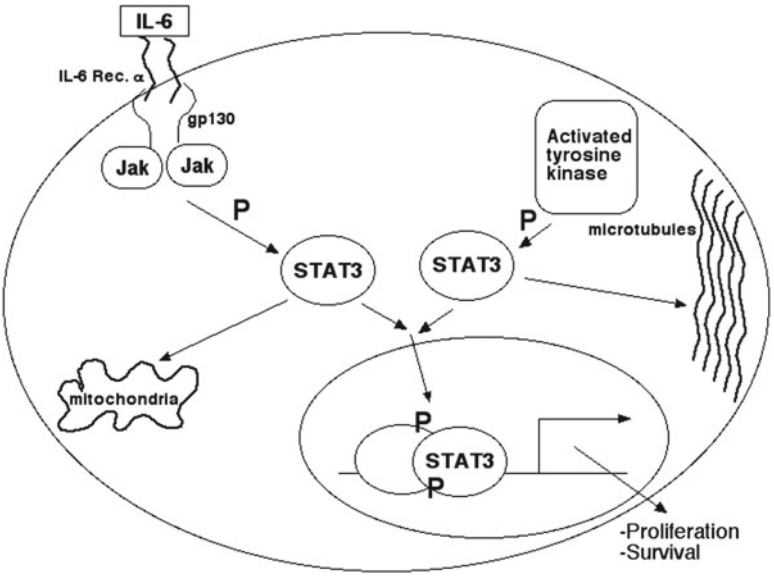


Fig. 7.1 STAT3 plays a central role in the biology of a multiple myeloma cell. In canonical STAT3 signaling, STAT3 is present in the cytoplasm of cells under basal conditions. Tyrosine phosphorylation of STAT3 can be induced by a cytokine such as IL-6, acting through Jak family kinases, or by a tyrosine kinase activated through mutation. This leads to the formation of STAT3 dimers which translocate to the nucleus, bind to specific nine base pair regions in the regulatory regions of target genes, and increase transcription of genes that regulate proliferation and survival. STAT3 may also play a role through interactions with mitochondria and microtubules

biological tools. Mutation of the critical tyrosine residue, tyrosine 705, or mutation of the DNA binding domain leads to STAT3 variants that not only lack activity themselves, but will inhibit the activation of wildtype endogenous STAT3 in a cell. These so-called dominant inhibitory forms of STAT3 have been very useful in discerning the role of STAT3 in mediating gene expression or a specific phenotype in a cancer cell. By contrast, the addition of two cysteine residues into STAT3 has generated a mutant form of STAT3, STAT3C, which forms dimers that are stabilized by the formation of intermolecular disulfide bonds [6]. Although this variant still requires tyrosine phosphorylation for its activity [7], it is constitutively active, and has been useful in dissecting cellular effects mediated by STAT3 activated in isolation [8]. A constitutively active form of STAT3 has not been reported to be found in human cancers, indicating that STAT3 acts as an oncogenic transcription factor not through its own mutation, but rather by conveying signals generated via mutations at upstream signaling points. Interestingly, familial forms of the hyper-IgE syndrome have been mapped to STAT3, and the mutations described are predicted to function as dominant inhibitory forms [9]. Since patients with this syndrome develop normally and are fertile, this "experiment of nature" suggests that inhibiting STAT3 in an adult should be tolerated well by normal cells, an important consideration for therapeutic development of STAT3 inhibitors.

7.1.2 Non-Canonical Mechanisms of STAT3 Function

While phosphorylation of the conserved carboxyl terminal tyrosine was clearly shown to be essential for nuclear translocation and the transcriptional function of STATs, other modifications of these proteins may occur as well. STAT3, as well as STAT1 and STAT4, contain a conserved carboxyl terminal serine residue (serine 727) that can be phosphorylated also. A number of serine, threonine kinases, including Erks and p38 MAP kinase can phosphorylate this site [10–12]. It was initially proposed that STAT3 serine phosphorylation modulates the magnitude of transcriptional activation mediated by tyrosine phosphorylated STAT3, or alters DNA binding. However, recent evidence suggests that serine phosphorylation of STAT3, which is commonly found in a variety of hematological and non-hematological malignancies, can directly activate transcription in the absence of tyrosine phosphorylation [13], or may modulate cellular function through localization within the mitochondria [14]. Unphosphorylated STAT3 may also associate with other transcription factors to modulate transcription, and may alter cellular function through non-transcriptional mechanism including interacting with the cytoskeleton [15–17]. Controversy still remains as to the relative importance of the transcriptional versus non-transcriptional functions of STAT3 in both normal cellular function and neoplastic transformation.

7.1.3 Jak-STAT Signaling

STAT3 can become activated by receptors for a variety of cytokines and growth factors, many of which signal through the activation of the Jak family of non-receptor tyrosine kinases. Jaks, or Janus kinases, were so-named for the two kinase domains encoded adjacent to each other in the protein, somewhat akin to the two-faced Roman god of doorways, Janus [18]. One of the domains is functionally inactive, and appears to function as an endogenous negative intramolecular regulator of the kinase activity of the other domain. The Jak family is comprised of four proteins, Jak1, Jak2, and Tyk2, all of which are expressed widely, and Jak3, whose expression is largely restricted to lymphocytes where it associates with the common gamma chain shared by cytokines such as IL-2, IL-7, and others. Jaks are cytoplasmic proteins that associate with the intracellular portion of cell surface receptors or receptor-associated proteins. For example, the receptors for cytokines such as IL-6, oncostatin M (OsM), ciliary neurotrophic factor (CNTF), and leukemia inhibitory factor (LIF) all are associated with a transmembrane protein called gp130. Jak1, Jak2, and Tyk2 can all associate with a cytoplasmic domain of gp130. When a cytokine of this family binds to its receptor, dimerization of the receptor and its associated proteins is induced. This brings the Jaks associated with gp130 into juxtaposition, thereby activating their tyrosine kinase activity. The activated Jaks transphosphorylate each other, as well as tyrosine residues on gp130 itself. These sites then allow the docking of STAT3, via its SH2 domain, triggering the phosphorylation and activation of STAT3.

Under physiological conditions, the phosphorylation of an inactive cytoplasmic STAT protein occurs within seconds of cytokine treatment. Phosphorylation reaches a peak generally between 15 and 60 min following stimulation, and STAT phosphorylation reaches baseline levels again within one to several hours. Given that the genes regulated by STAT3 control such critical cellular processes as cell cycle progression, survival, and self-renewal, it is essential that regulation of these genes be tightly controlled. In fact, STAT3 induces expression of a number of negative regulators which block further phosphorylation of STAT3, and promote its dephosphorylation and degradation [19].

7.1.4 The Role of Coactivation of STAT1

Many cytokines that can cause the activation of STAT3 also activate the phosphorylation of the related STAT family member, STAT1. STAT1 was originally identified as a mediator of the effects of interferons [20, 21]. Like interferons, STAT1 activation can mediate cell cycle arrest and a lowered threshold for apoptosis. When STAT1 and STAT3 are activated contemporaneously, it is possible to detect STAT1 homodimers, STAT3 homodimers, and STAT1–STAT3 heterodimers. The relative activation of each STAT, and the genomic localization of each dimer may determine the biological response of a cell to cytokines that signal though both of these proteins.

7.1.5 Analyzing STAT Activation in Clinical Samples

A number of methods have been developed to follow STAT3 activation in primary patient samples including paraffin-embedded samples. Antibodies have been developed that recognize the tyrosine phosphorylated form of STAT3 [22]. These can be used to measure STAT3 phosphorylation by immunoblot (western blot). While this can provide quantitative information about levels of STAT3 phosphorylation in a sample, given the admixture of nonmalignant cells, including stroma, immune, inflammatory, blood and vascular cells, interpretation of such findings must be viewed cautiously. Similarly, nuclear or whole cell extracts can be prepared from samples and analyzed for the ability to bind DNA probes containing canonical STAT3 binding sites by electrophoretic mobility shift assays (EMSAs). Antibodies specific for the phosphorylated form of STAT3 can also be used to analyze cells by immunofluorescence, immunohistochemistry, or flow cytometry, all of which can provide information on the proportion of cells with STAT3 activation within the tumor cell population specifically. Finally, antibodies to total STAT3 can be used for immunostaining, in which nuclear localization can be used as a marker for STAT activation. These methods may be less likely to discern a role for STAT3 operating through a non-canonical pathway such as within the mitochondria or cytoskeleton.

Given the transient nature of STAT3 phosphorylation during physiological signaling, analysis of blood cells or bone marrow from healthy donors generally fails to show evidence of STAT activation. By contrast, analysis of cells from a variety of tumor types, both hematological and non-hematological, have shown evidence for constitutive activation, i.e., tyrosine phosphorylation, of STAT3 [23, 24]. In particular, analysis of primary cells from patients with myeloma, as well as myeloma cell lines, has consistently revealed a high proportion of samples in which STAT3 is prominently activated [25]. This raises two immediate questions: what is the mechanism driving the continued activation of STAT3 in multiple myeloma, and what is the consequence to cellular function of persistent activation of this transcription factor.

Constitutive phosphorylation of STATs occurs widely in cancer, though the mechanism driving this phosphorylation is not always clear. Among hematological cancers, the constitutive activation of STAT5, for example, was first described in chronic myelogenous leukemia (CML), and it could be shown that the Bcr-Abl fusion tyrosine kinase could directly contribute to this phosphorylation [26, 27]. Similarly, other oncogenic tyrosine kinases arising from chromosomal translocations have been found to drive chronic STAT phosphorylation [28–30]. In multiple myeloma, there has been little evidence for common activating mutations in tyrosine kinases. However, it has been clear for many years that myeloma cells are dependent on the presence of cytokines, particularly IL-6 for continued growth [31–34]. IL-6 may be generated by the myeloma cell itself, thereby acting in an autocrine mechanism [35]. Alternatively, IL-6 may be produced by bone marrow stromal cells, perhaps induced by the myeloma cells, and thereby promote STAT3 activation in the myeloma cells through a paracrine mechanism [36–38]. In some situations, STATs may be activated by direct cell–cell contact [39], and thus bone marrow stromal cells could promote STAT3 phosphorylation either through soluble factors, direct contact, or both. Since STAT3 activation generally mediates a pro-survival effect in hematopoietic cells, the fact that bone marrow stromal cells can induce STAT3 phosphorylation is consistent with their role in promoting myeloma cell survival [40, 41]. This also explains the finding that myeloma cell lines are more sensitive to the cytotoxic effects of chemotherapy or radiation when grown in isolation versus their response in the presence of stromal cells. Similarly, performing cytotoxic sensitivity assays on myeloma cells grown in culture often predicts greater sensitivity of the cells to drugs than what is observed in vivo.

Another implication of the prominent role of IL-6 autocrine and paracrine loops in multiple myeloma concerns the central role played by Jak kinases. Jaks, particularly Jak2, is activated through point mutations in a large fraction of myeloproliferative diseases, particularly polycythemia vera. This finding has generated a large amount of interest in developing inhibitors of Jak2 as a form of targeted therapy for these diseases. In animal studies and early clinical investigations, it appears that these Jak inhibitors are fairly well tolerated. In the IL-6 autocrine and paracrine loops found in myeloma, Jak family members play a key role in driving STAT3 activation. Consistent with this role, pharmacological inhibition of Jaks can decrease STAT3 phosphorylation and decrease the survival and proliferation of

myeloma cells [42, 43]. These observations provide further support to the concept that targeting key signaling intermediates driving the malignant phenotype, even if they themselves are not mutated, may be a powerful therapeutic strategy.

7.1.6 Inactivation of Negative Regulators of STAT3

As noted, incubation of normal cells with cytokines generally leads to a transient activation of STAT3 phosphorylation, as the initial stimulation of phosphorylation diminishes following the activation of negative regulators of this pathway. This raised the possibility that the continued prominent phosphorylation of STAT3 seen in myeloma cells may be a combination of cytokine-induced activation of Jak kinases, accompanied by a downregulation of one or more negative regulators of STAT3 signaling. In fact, key negative regulators of STAT3 phosphorylation, including the phosphatase SHP1 or SOCS3, are often inactivated in myeloma cells through mechanisms such as DNA methylation [44, 45].

7.1.7 STAT3 in the Pathogenesis of Myeloma

The second key question that arises from the observation that continued STAT3 activation is a common event in myeloma cells is whether this directly contributes to the pathogenesis of this cancer, or merely reflects a bystander activation of this transcription factor with little physiological consequence. To address this issue, a number of experimental approaches have been pursued. Using dominant inhibitory forms of STAT3, or RNA interference targeting this protein, it has been shown that cellular proliferation and survival of myeloma cells is strongly diminished following inactivation of STAT3 [25, 46–48]. These experiments have provided strong evidence that STAT3 activation is not a byproduct of malignant transformation of plasma cells, but rather represents a central mediator of the neoplastic process in multiple myeloma. This finding coupled with evidence that STAT3 inhibition is well tolerated in normal cells, has suggested that STAT3 inhibition would represent an excellent therapeutic target in myeloma, with the potential of displaying a very high therapeutic index.

7.2 STAT3 Target Genes

That STAT3 is a key mediator in the pathogenicity of multiple myeloma is not surprising, as STAT3 regulates the transcription of critical target genes involved in survival, differentiation, migration, angiogenesis, and apoptosis. STAT3 not only directly regulates transcription by binding to STAT3 consensus sites within the

genome, but it also upregulates genes indirectly by modulating the expression of additional transcription factors including Fos and Jun. While it had been challenging to identify key direct STAT3 target genes in the past, gene expression microarrays have been an invaluable tool in addressing this issue [8]. In addition, identification of STAT binding sites using homology and computational approaches [49–51] or chromatin immunoprecipitation (ChIP) combined with microarrays or sequencing has greatly enhanced our ability to define direct STAT target genes [52, 53]. As more of these studies are conducted, it will be possible to define a STAT3 specific gene expression pattern in multiple myeloma that may be useful in providing prognostic and predictive information for individual patients. To date, numerous STAT3 target genes have been identified using these and other methods. A few key target genes that have important functions in multiple myeloma will be highlighted.

7.2.1 Mcl-1

One of the best described target genes of STAT3 is Mcl-1 (myeloid cell leukemia-1). Mcl-1 is a member of the BCL2 family of anti-apoptotic proteins whose function is to bind to BH3-only pro-apoptotic proteins, thereby preventing oligomerization of Bax or Bak and eventual cytochrome c release [54]. Unlike most BCL2 family members, Mcl-1 has a short half life, so changes in expression either at the level of transcription or translation can rapidly affect cellular survival. STAT3 upregulates Mcl-1 expression by binding directly to the STAT3 regulatory region of the Mcl-1 gene [55]. Myeloma cells thus become dependent on STAT3 and Mcl-1 for survival most likely due to an oncogene addiction mechanism [56]. Constitutive expression of Mcl-1 promotes resistance to chemotherapy by providing a continuous survival signal and inhibiting the expression levels of Mcl-1 promotes apoptosis of myeloma cells. Therefore, one therapeutic strategy to decrease the expression levels of Mcl-1 is through the inhibition of STAT3 activation by targeting of the IL-6/JAK/STAT3 pathway.

Bcl-xl and Bcl-2 are additional anti-apoptotic proteins which are also STAT3 targets. These two proteins also have similar functions as Mcl-1 such as binding to pro-apoptotic proteins, though their protein half-lives are much longer. In multiple myeloma cell lines that have constitutive activation of STAT3, these genes are also upregulated, giving these cells additional survival signals. Upregulation of Bcl-xl was shown to prevent Fas mediated apoptosis in U266 cells in a STAT3 dependent manner [25]. Thus, these findings make clear that STAT3 upregulates many genes promoting cell survival. To determine which survival protein in particular a cell is depending on, one can perform BH3 profiling, a technique that can identify which pro-survival gene is directly preventing apoptosis in the cancer cell [57]. Drugs that bind to these survival proteins are currently being developed and used in clinical trials for a variety of cancers, and they may be useful for multiple myeloma therapy [57]. Targeting STAT3 activation and function alone or in combination with a specific inhibitor of a key survival gene, may provide a particularly useful combination therapy.

7.2.2 VEGF

The STAT3 target gene VEGF (vascular endothelial growth factor) plays a number of important roles in multiple myeloma. IL-6 activation of STAT3 results in upregulation of VEGF expression. Secretion of this growth factor then causes a number of signaling events to occur. VEGF secretion leads to IL-6 upregulation and secretion in the bone marrow stromal cells and endothelial cells, leading to paracrine activation of STAT3 and ultimately increased expression of Mcl-1 and other STAT3 target genes [58]. Therefore, VEGF indirectly promotes the survival of myeloma cells. In addition, VEGF secretion affects the cells in the bone marrow microenvironment, promoting angiogenesis [59]. The amount of vasculature of the bone marrow is a prognostic indicator for myeloma [60], and some of the current therapies for multiple myeloma, such as thalidomide, are employed in part to inhibit angiogenesis [61]. Targeting STAT3 should therefore not only prevent the survival signals in the myeloma cells but also reduce the amount of angiogenesis in the bone marrow microenvironment.

7.2.3 BCL3

BCL3 expression transforms NIH3T3 cells and induces tumor growth in mice [62]. In multiple myeloma, BCL3 is associated with increased proliferation, and high expression of BCL3 at time of diagnosis leads to a poor prognosis [63]. However, in vitro work has not substantiated these findings since forced overexpression of BCL3 in myeloma cell lines leads to increased apoptosis, while loss of BCL3 expression has little effect on cell viability [64]. Therefore, the exact role that BCL3 plays in multiple myeloma remains to be determined. BCL3 is a member of the IκB family, and BCL3 can both positively and negatively affect NF-κB transcriptional activity depending on the context [65, 66]. BCL3 has been shown to be upregulated by IL-6 and STAT3 in multiple myeloma and the STAT3 binding site in the BCl3 gene has been identified [64]. Regulation of BCL3 by STAT3 highlights the potential intersection of the STAT3 and NF-κB pathways in multiple myeloma.

7.2.4 BCL6

The transcriptional repressor BCL6 has been described as having a prominent oncogenic function in diffuse large B cell lymphoma (DLBCL) and in preventing B cell differentiation. BCL6 has been shown to be expressed commonly in myeloma patient samples, and it promotes survival in multiple myeloma cell lines [67]. This is particularly true in myeloma cells growing in the presence of bone marrow stromal cells [68], most likely due to IL-6 secretion and resultant STAT3 activation. BCL6

is regulated directly by STAT3 binding to its promoter region, and the key STAT binding sites have been identified by analyzing inter-species conservation of sequence homology [3, 67, 69]. This raises the possibility that STAT3 is having a wide effect on gene expression due to upregulation of a transcriptional repressor such as BCL6, which will modulate the expression of additional target genes. Importantly, using RNA interference to knock down BCL6 in cells containing constitutively active STAT3 resulted in decreased growth and increased sensitivity to chemotherapeutic drugs [67, 68]. This suggests that treating myeloma cells with a BCL6 inhibitor in combination with a STAT3 inhibitor may be a useful combination for myeloma therapy.

7.2.5 Mir-21

It is becoming increasingly clear that in addition to regulating mRNA expression, STATs play an important role in modulating expression of micro RNAs as well. The first micro RNA identified as a STAT3 target was Mir-21, and the STAT3 binding site of Mir-21 was initially identified in multiple myeloma using sequence homology [33]. Like many of the STAT3 target genes, Mir-21 is involved in the control of apoptosis. Mir-21 targets a number of genes involved in apoptosis, such as the tumor suppressors p53 and PTEN, and proapoptotic genes involved in cytochrome c release [70]. Mir-21 is upregulated in patients that have multiple myeloma [71]. In multiple myeloma cell lines, Mir-21 expression promotes survival by reducing the dependency on IL-6 in INA-6 and XG-1, cell lines that require IL-6 for survival [33]. This suggests that upregulation of Mir-21 by STAT3 is another mechanism by which STAT3 promotes survival, via Mir-21 mediated inhibition of proapoptotic protein expression.

7.2.6 Biologic Significance of STAT3 Activation in Myeloma

In reviewing STAT3 target genes that are known to be significantly expressed in multiple myeloma, it is striking to see how many are involved in survival and protection from apoptosis. While this seems to be a major function of STAT3 in myeloma, it is by no means the only function that STAT3 plays in myeloma. STAT3 upregulates VEGF which does promote survival of myeloma cells indirectly; however, VEGF also promotes angiogenesis. Thus, the STAT3 activation of myeloma cells leads to secretion of VEGF which affects the bone marrow microenvironment in which the myeloma cells are located. In addition to the effects on angiogenesis, STAT3 target genes may also affect the activity of NF-κB, another important transcription factor in myeloma. Upregulation of BCL3 by STAT3 may both promote and inhibit NF-κB function on specific genes. Understanding the functional interplay between STAT3, BCL3, and NF-κB may lead to new insights into the pathogenesis of myeloma.

Reflecting the large number of genes promoting protection from apoptosis, it is not surprising that IL-6 and STAT3 have been implicated in resistance to many chemotherapeutic drugs. The target genes Mcl-1, Bcl2, and Bcl-xl have all been shown to promote resistance to cytotoxic agents. In fact, it has been shown that inhibiting STAT3 with the Jak2 kinase inhibitor AG490 sensitizes U266 cells to a variety of chemotherapeutic agents, such as cisplatin, fludarabine, and vincristine [41]. Treatment with AG490 led to a decrease in Bcl-xl protein expression. This suggested that Bcl-xl may be promoting resistance to chemotherapy and that if its expression is reduced, cells can be rendered sensitive to chemotherapeutic drugs. Therefore, targeting STAT3 for the treatment of myeloma may be a useful form of therapy alone or in combination with chemotherapy utilized currently for myeloma therapy.

7.3 STAT Inhibition as a Therapeutic Strategy in Myeloma

Given the critical contribution of STAT3 in myeloma biology, it is likely that targeting STAT3 would be a useful treatment strategy. Because of this, a great deal of effort has gone into the identification of molecules that can block STAT3 function at every step from its phosphorylation to its recruitment of transcriptional coactivators (Fig. 7.2). A greater understanding of the role of STAT3 in myeloma, as well as further refinement of STAT3 inhibitors, has great potential for advances in the clinic, and several approaches to targeting this transcription factor will be highlighted.

7.3.1 Kinase Inhibitors

In cancer, constitutive activation of STATs often occurs through activating mutations in an upstream tyrosine kinase. Thus, targeting the kinase will indirectly reduce STAT signaling. Since tyrosine kinases are activated in many tumor types, great effort has gone into identification of pharmacological kinase inhibitors. The kinase thought to be most responsible for activating STAT3 in myeloma is Jak2. Though STAT activation in some myeloproliferative disorders (MPD) occurs through an activating mutation in Jak2, V617F, the activation of Jak2 in myeloma appears to be through IL-6 signaling, a cytokine which can be produced by the myeloma cells themselves or secreted by bone marrow stromal cells (BMSC). Since Jak2 mutations are critical in myeloid malignancies, the major focus of developing Jak2 inhibitors has been in these tumors. One example is TG101348, which was synthesized by structure based drug design [72]. Treatment of MPD cells with TG101348 reduced their viability, and decreased STAT5 phosphorylation, the principal endpoint of Jak2V617F signaling. Therefore, inhibition of Jak2 signaling may be useful in patients having myeloid malignancies associated with mutant Jak2.

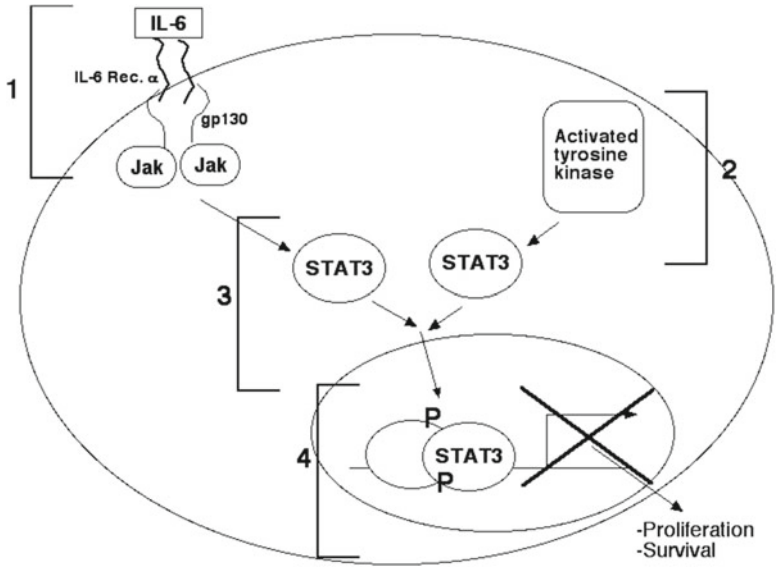


Fig. 7.2 Inhibiting STAT3 is an attractive therapeutic approach with the potential for a high therapeutic index. Although many strategies are being pursued, they fall under four main categories (1) Inhibition of cytokine-mediated STAT3 phosphorylation (such as inhibition of IL-6, the IL-6 receptor chains or Jak family kinase, or enhancing expression of negative regulators such as SOCS proteins); (2) inhibition of other activated tyrosine kinases, such as Src family members; (3) targeting STAT3 to block its activation, including inhibition of the STAT3 SH2 domain or nuclear translocation; and (4) inhibiting the transcriptional function of STAT3 by blocking DNA binding or co-activator recruitment

Two recent publications highlight the utility of inhibiting Jak2 in myeloma. INCB20 and INCB16562 are two synthetic compounds that were identified as Jak2 inhibitors [73, 74]. Both compounds showed a dose dependent decrease in the viability of myeloma cells containing constitutive Jak2 signaling such as the IL-6 dependent INA-6 cell line, whereas cells that do not have activated Jak2, such as MM.1s, were largely unaffected. Jak2 inhibition overcomes the survival advantage given by co-culture with bone marrow stromal cells demonstrating that these drugs may be effective in the pro-survival environment in which myeloma cells exist in patients.

MM.1s cells are sensitive to dexamethasone, a synthetic glucocorticoid commonly used for the treatment of myeloma. However, IL-6 protects MM.1s cells from dexamethasone induced cell death. Reflecting the fact that Jak2 mediates IL-6 signaling, treatment of IL-6 stimulated MM.1s cells with INCB20 or INCB16562 resulted in reduced survival. This reduction in cell survival correlated with reduced activation of SHP-2, AKT, MAPK, STAT1, and STAT3, all components of Jak2 signaling in myeloma cells. Therefore, therapeutic targeting of Jak2 in myeloma may be a useful therapy in cells sensitive or resistant to conventional chemotherapy.

A recent study used a different approach to identify a STAT3 inhibitor [75]. Since the endpoint of STAT3 signaling is transcriptional activation, an assay was developed that measured changes in STAT3-dependent gene expression. A STAT3 responsive promoter linked to a luciferase reporter gene was stably transfected into STAT1 null cells. In this system, STAT3 activity could be specifically modulated using IL-6 without concern of confounding effects from the simultaneous activation of STAT1. To circumvent the challenges of bringing novel small molecule inhibitors to the clinic, a library of drugs with known safety characteristics was interrogated for STAT3 inhibitory activity. A number of compounds were identified as STAT3 inhibitors, including the anti-bacterial drug nifuroxazide, a drug not previously known to have anti-tumor activity. When myeloma cells were cultured with nifuroxazide, viable cell number was reduced, even in the presence of BMSC. This reduction in cell survival was correlated with a reduction in STAT3 tyrosine phosphorylation and a reduction in the expression of the STAT3 target gene Mcl-1. In addition, the tyrosine phosphorylation of two Jak family members, Jak2 and Tyk2, was reduced, suggesting that the mechanism of action of nifuroxazide is through Jak inhibition. This method of identifying STAT3 inhibitors may yield other, more potent inhibitors that may reach clinical use.

A number of natural compounds have recently been identified as having anti-myeloma activity, which may be mediated through inhibition of STAT3. One example is curcumin, which is derived from tumeric, a spice used in Indian cuisine and folk medicine [76]. Curcumin was shown to have Jak2 inhibitory activity, but the high dose required for efficacy suggested that this compound might not be useful in the clinic. To overcome these limitations, molecular modeling was employed and it was determined that curcumin may bind to the STAT3 SH2 domain, thereby preventing STAT3 from being recruited and activated by Jak2 [77]. However, this binding theoretically only occurs when curcumin is in the keto form, though it is almost entirely in the enol form in solution. By modifying curcumin to reside primarily in the keto form, a new molecule called FLLL32 was characterized. FLLL32 reduced Jak2 and STAT3 phosphorylation in a number of cancers, including U266 myeloma cells, and this correlated with loss of cell viability. These results demonstrate that combining modern molecular modeling with folk medicine may generate new and effective therapies for many tumor types, including myeloma.

7.3.2 IL-6 as a Therapeutic Target

Since kinases can be constitutively activated in many tumors, much recent effort has focused on the direct targeting of these proteins. In some cases, the kinase itself is mutated to a constitutively activated form. However, non-mutant kinases are often constitutively activated in cancers by cytokines, such as the activation of Jak2 by IL-6 in myeloma. IL-6 is a known survival factor for myeloma cells, and serum levels of the soluble IL-6 receptor have some correlation with reduced survival [78]. Therefore, targeting tyrosine kinase signaling upstream of the kinase activity may prove useful as a treatment option.

There have been numerous studies over the years testing the effect of monoclonal antibodies to IL-6 on myeloma cell survival. These have shown mixed results. There are convincing data showing the role of IL-6 in myeloma survival using both in vitro cell models and in vivo using mouse models, and recent clinical results in patients have shown promise. Patients given a murine monoclonal antibody directed against IL-6 showed some response [79]. Though these data strongly suggest that IL-6 monoclonal antibody therapy may be useful, patients generally did not achieve a complete response. This may be due to the short half-life of the antibody in serum, which is related to the immunogenicity of the murine components of the antibody.

To overcome these limitations, a fully humanized monoclonal antibody, mAb 1339, was generated and tested using cell culture and murine models [80]. mAb 1339 reduced the viability of the IL-6 dependent myeloma cell lines INA-6 and XG1, while reducing the survival advantage given by bone marrow stromal cells, including the IL-6 responsive cell line MM.1s. These results were correlated with a decrease in the pathways downstream of IL-6, including MAPK, AKT, and STAT3. Significantly, nearly peak levels of mAb 1339 remained for at least a week after a single injection of this antibody in a murine model of myeloma. Therefore the effect of once weekly mAb 1339 injections was determined in this model, in which INA-6 myeloma cells are implanted in a human bone chip in immunodeficient mice. Tumor burden was reduced after 4 weeks in mAb 1339 treated mice compared to control animals. Therefore, these results suggest that mAb 1339 may be useful in human patients with myeloma, and it would be important to correlate clinical response with reduction in STAT3 activity.

7.3.3 Targeting the IL-6 Receptor

While inhibiting IL-6 directly shows promise, an alternative strategy is to target the IL-6 receptor. There are two central components to the IL-6 receptor: the ligand-binding IL-6 receptor α chain (CD126), and the signal transducing gp130 component (CD130). The gp130 molecule binds to the Src family kinase Hck, which then may serve as an adaptor protein activating the MAPK and PI3K pathways. Though Hck can activate STATs in some cells, it does not activate STAT3 in myeloma. Therefore, it is not surprising that when the acidic domain of gp130, which mediates its association with Src family kinases, was inhibited using a blocking peptide, there was no loss of STAT3 activation, though there was a loss of MAPK and PI3K activity and a loss of cell viability [81]. This demonstrates the specificity by which the components of the IL-6 receptor affect particular signaling pathways, and indicates that non-STAT pathways activated by IL-6 may also play an important role in promoting myeloma cell survival. It is possible that targeting the IL-6 receptor α chain will be useful for inhibiting STAT3 signaling in myeloma. In fact, the histone deacetylase inhibitor ITF2357, which shows cytotoxic activity in myeloma, reduced expression of the IL-6 receptor α chain, and decreased IL-6 induced STAT3 activation [82]. Therefore, targeting the IL-6 receptor may be useful as a therapeutic target for myeloma patients.

7.3.4 Modulating Negative Regulators of STAT3

In normal cells, cytokine signaling is transient, with the phosphorylation of STAT proteins occurring within minutes and reaching a maximum at 15–60 min. This carefully controlled physiologic STAT activation occurs through the tight regulation of cytokine signaling by negative regulators, such as the suppressor of cytokine signaling (SOCS) family. This contrasts with STAT signaling in tumor cells, where the activation of STAT proteins is often prolonged or continuous. In tumors, STATs may be phosphorylated by a kinase that is activated through mutation, or that is activated by autocrine or paracrine loops involving cytokines such as IL-6. However, there is increasing evidence that many tumors also contain decreased expression or activity of negative regulators, which contributes to the prolonged activation of STATs. This may be the sole STAT activating event, or it may occur in combination with constitutive tyrosine kinase activation. Since inactivation of negative regulators promotes the constitutive activation of STATs, the reactivation of these tumor suppressors may provide an effective therapeutic option.

There are three main types of negative regulators of STATs. The SOCS proteins have SH2 phosphotyrosine-binding domains that compete with the binding of STATs to activated tyrosine kinases. They also can recruit ubiquitination enzymes that lead to the degradation of the tyrosine kinase. The second group of negative regulators are the protein inhibitor of activated STAT (PIAS) proteins, which may affect signaling through SUMOylation of STATs. The third type of negative regulators are phosphatases, which remove the activating phosphorylation of STATs. Each of these negative regulators inhibits the continual signaling of STATs. Since these genes are often induced by activated STATs, they serve as negative feedback loops of the STAT-signaling pathway.

One mechanism of inactivating tumor suppressors is to downregulate their expression, and this may happen by methylation of their regulatory regions. Indeed, analysis of methylation of the promoter region of SOCS-1 revealed hypermethylation in the two myeloma cell lines tested, as well as 62 % of the primary patient samples [45]. The myeloma cell lines contain activated STAT3, though the activation status of STAT3 was not examined in the patient samples. It is also unknown if there is a direct functional link between hypermethylation of the SOCS promoter and the tumorigenic properties of the cells. However, a similar study in hepatocellular carcinogenesis showed that reexpression of SOCS-1 in a cell line containing a silenced endogenous SOCS-1 gene resulted in reduced growth in soft agar, suggesting that a similar effect may be seen in myeloma [83]. In a more recent study, SOCS-1 was reexpressed in a variety of myeloma cell lines. The authors found that IL-6 dependent cell lines tended to show reduced cell viability when SOCS-1 was reexpressed, while IL-6 independent cells lines were unaffected [84]. However, these intriguing results raise several interesting issues. It will be important to follow up with an analysis of the effect of the ectopic expression of SOCS-1 on tyrosine phosphorylated STAT3, which will help elucidate the functional consequence of SOCS-1. Furthermore, the expression of SOCS-1 in these cell lines needs to be

clarified, since U266 cells have been reported to have a hypermethylated SOCS-1 promoter [45], though expression of SOCS-1 had no effect in these cells [84]. Lastly, a follow up study concluded that there was no correlation between SOCS-1 expression and clinical outcomes [85]. Though more work needs to be done, it is likely that SOCS-1 silencing plays a role in the pathogenesis of myeloma, and that reexpressing SOCS-1 by gene therapy or with demethylating agents could be useful in the treatment of myeloma.

Several current therapies for myeloma are known immune system modulators, such as lenalidomide and pomalidomide. A recent study has shown that these drugs have the effect of modulating SOCS-1 expression in both the immune cells which are involved in cell mediated killing, as well as in the myeloma cells themselves [86]. This is notable in that STAT3 activation in immune cells may lead to immune dysfunction, and inhibition of STAT3 may enhance anti-tumor immunity [87]. Both of these drugs induced SOCS-1 expression in myeloma cells, which can result in reduced STAT3 activation and reduced survival. Therefore, at least part of the effect of the immune modulators lenalidomide and pomalidomide may involve inducing the expression of SOCS-1.

SHP-1 is a phosphatase that may play a role in myeloma pathogenesis. The promoter of SHP-1 has been analyzed in myeloma patient samples, and methylation was found in almost 80 % of the patients, as well as in the U266 myeloma cell line [44]. To determine if there was a functional significance of this methylation, U266 cells were treated with the DNA demethylating agent 5-azacytidine. After several days, SHP-1 expression increased, while the phosphorylation of STAT3 concomitantly decreased, though it was not reported what effect this treatment had on U266 cell viability. In patients, there was a slight correlation between survival and SHP-1 methylation status. Those patients having a methylated SHP-1 promoter were more likely to have a worse prognosis, raising the possibility that these patients may have more aggressive tumors due to enhanced STAT3 signaling. Therefore, it is likely that SHP-1, and possibly other phosphatases, serve as tumor suppressors in myeloma, and that reexpressing these phosphatases may increase the survival of myeloma patients.

7.3.5 Additional Therapeutic Strategies

Given the role of STATs in transducing signals from cytokines, much of the early work on STAT biology was performed in hematopoietic cells [21, 88]. Similarly, a focus on constitutive activation of STATs in cancer pathogenesis focused initially on many hematologic malignancies, including multiple myeloma [89]. However, it is now clear that STAT activation, particularly STAT3 activation, is a common event in many forms of non-hematologic cancers as well [90], and STAT3 has become a focus for a number of novel therapeutic strategies. In addition to the approaches discussed, a number of additional approaches are being utilized. For example, medicinal chemistry approaches are being utilized to target the SH2 domain of

STAT3, which would block both recruitment to activated kinases as well as dimerization [91, 92]. Although the DNA binding domain of a STAT dimer represents a large surface that might not seem amenable to classic small molecule inhibitors, novel strategies are being utilized to block the interaction between STATs and DNA. For example, decoy oligonucleotides, short double stranded oligonucleotides containing a STAT binding site, have been shown to be effective at serving as an intracellular sink for activated STATs, thereby attenuating their transcriptional function [93–95]. It has also been possible to synthesize polyamides [96] which can bind to DNA in a sequence specific manner, thereby blocking the function of a transcription factor. Finally, there is evidence that recruitment of co-activators by STAT3 might be blocked by specific small molecules, thereby inhibiting the ability of STAT3 to initiate transcription. Although it is too early to assess the clinical applicability of some of these approaches, it is clear that the increase in molecular understanding of myeloma pathogenesis has provided insight into therapeutic strategies that may be very valuable.

7.4 Conclusion

Although myeloma is generally viewed as an incurable malignancy, the dramatic increase in our understanding of the molecular pathogenesis of this cancer has led to the identification of several key rational targets for therapeutic intervention. Since the pattern of gene expression in a myeloma cell is largely responsible for its malignant behavior, the targeting of oncogenic transcription factors, particularly STAT3, holds promise for providing treatment strategies with a very high therapeutic index. Although transcription factors had not traditionally been thought of as tractable targets for therapeutic inhibition, increasing evidence has shown that these proteins can be targeted specifically, and that by decreasing expression of key target genes, they can alter the biology of myeloma cells. Clinical trials of STAT3 inhibitors have been initiated in other malignancies, and thus there is increased hope that these agents, alone or in combination with current strategies, may greatly enhance the outcome of patients with this prevalent disease.

References

1. Darnell JE Jr (2002) Transcription factors as targets for cancer therapy. Nat Rev Cancer 2(10):740–749
2. Ihle JN (2001) The Stat family in cytokine signaling. Curr Opin Cell Biol 13(2):211–217
3. Walker SR, Nelson EA, Frank DA (2007) STAT5 represses BCL6 expression by binding to a regulatory region frequently mutated in lymphomas. Oncogene 26(2):224–233
4. Haan S, Kortylewski M, Behrmann I, Müller-Esterl W, Heinrich PC, Schaper F (2000) Cytoplasmic STAT proteins associate prior to activation. Biochem J 345(Pt 3):417–421
5. Bhattacharya S, Schindler C (2003) Regulation of Stat3 nuclear export. J Clin Investig 111(4):553–559

6. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C et al (1999) *Stat3* as an oncogene. Cell 98:295–303
7. Liddle FJ, Alvarez JA, Poli V, Frank DA (2006) Tyrosine phosphorylation is required for functional activation of disulfide-containing constitutively active STAT mutants. Biochemistry 45:5599–5605
8. Alvarez JV, Febbo PG, Ramaswamy S, Loda M, Richardson A, Frank DA (2005) Identification of a genetic signature of activated signal transducer and activator of transcription 3 in human tumors. Cancer Res 65(12):5054–5062
9. Holland SM, DeLeo FR, Elloumi HZ, Hsu AP, Uzel G, Brodsky N et al (2007) STAT3 mutations in the hyper-IgE syndrome. N Engl J Med 357(16):1608–1619
10. Turkson J, Bowman T, Adnane J, Zhang Y, Sekharam M, Frank DA et al (1999) Requirement of Rac-1 mediated p38 and JNK signaling for Stat3 transcriptional activity induced by the Src oncoprotein. Mol Cel Biol 19(11):7519–7528
11. Chung J, Uchida E, Grammer TC, Blenis J (1997) STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. Mol Cell Biol 17(11):6508–6516
12. Xu B, Bhattacharjee A, Roy B, Xu H-M, Anthony D, Frank DA et al (2003) IL-13 induction of 15-lipoxygenase expression requires p38 MAPK-mediated serine 727 phosphorylation of STAT1 and STAT3. Mol Cell Biol 23:3918–3928
13. Hazan-Halevy I, Harris D, Liu Z, Liu J, Li P, Chen X et al (2010) STAT3 is constitutively phosphorylated on serine 727 residues, binds DNA, and activates transcription in CLL cells. Blood 115(14):2852–2863
14. Gough DJ, Corlett A, Schlessinger K, Wegrzyn J, Larner AC, Levy DE (2009) Mitochondrial STAT3 supports Ras-dependent oncogenic transformation. Science 324(5935):1713–1716
15. Yang J, Stark GR (2008) Roles of unphosphorylated STATs in signaling. Cell Res 18(4):443–451
16. Walker SR, Chaudhury M, Nelson EA, Frank DA (2010) Microtubule-targeted chemotherapeutic agents inhibit STAT3 signaling. Mol Pharmacol 78(5):903–908
17. Germain D, Frank DA (2007) Targeting the cytoplasmic and nuclear functions of STAT3 for cancer therapy. Clin Cancer Res 13:5665–5669
18. Ghoreschi K, Laurence A, O'Shea JJ (2009) Janus kinases in immune cell signaling. Immunological Rev 228(1):273–287. doi:10.1111/j.1600-065X.2008.00754.x
19. Alvarez JV, Frank DA (2004) Genome-wide analysis of STAT target genes: elucidating the mechanism of STAT-mediated oncogenesis. Cancer Biol Ther 3:1045–1050
20. Pellegrini S, John J, Shearer M, Kerr IM, Stark GR (1989) Use of a selectable marker regulated by [{alpha}] interferon to obtain mutations in the signaling pathway. Mol Cell Biol 9:4605–4612
21. Darnell JE Jr, Kerr IM, Stark GR (1994) Jak-STAT pathway and transcription activation in response to IFNs and other extracellular signaling proteins. Science 264:1415–1420
22. Bonni A, Sun Y, Nadal-Vicens M, Bhatt A, Frank DA, Rozovsky I et al (1997) Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science 278:477–483
23. Frank DA (2003) STAT signaling in cancer: Insights into pathogenesis and treatment strategies. Cancer Treat Res 115:267–291
24. Yu H, Jove R (2004) The STATs of cancer–new molecular targets come of age. Nat Rev Cancer 4(2):97–105
25. Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R et al (1999) Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity 10:105–115
26. Frank DA, Varticovski L (1996) BCR/abl leads to the constitutive activation of Stat proteins, and shares an epitope with tyrosine phosphorylated Stats. Leukemia 10:1724–1730
27. Carlesso N, Frank DA, Griffin JD (1996) Tyrosyl phosphorylation and DNA-binding activity of STAT proteins in hematopoietic cell lines transformed by Bcr/Abl. J Exp Med 183:811–820
28. Schwaller J, Parganas E, Wang D, Cain D, Aster JC, Williams IR et al (2000) Stat5 is essential for the myelo- and lymphoproliferative disease induced by TEL/JAK2. Mol Cell 6:693–704

29. Sternberg DW, Tomasson MH, Carroll M, Curley DP, Barker G, Caprio M et al (2001) The TEL/PDGFbetaR fusion in chronic myelomonocytic leukemia signals through STAT5-dependent and STAT5-independent pathways. Blood 98(12):3390–3397
30. Wilbanks AM, Mahajan S, Frank DA, Druker BJ, Gilliland DG, Carroll M (2000) TEL/PDGFβR fusion protein activates STAT1 and 5: a common mechanism for transformation by tyrosine kinase fusion proteins. Exp Hematol 28:584–593
31. Klein B, Zhang XG, Yang LZ, Bataille R (1995) Interleukin-6 in human multiple myeloma. Blood 85:863–874
32. Levy Y, Tsapis A, Brouet JC (1991) Interleukin-6 antisense oligonucleotides inhibit the growth of human myeloma cell lines. J Clin Invest 88:696–699
33. Loffler D, Brocke-Heidrich K, Pfeifer G, Stocsits C, Hackermuller J, Kretzschmar AK et al (2007) Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. Blood 110(4):1330–1333
34. Puthier D, Bataille R, Amiot M (1999) IL-6 up-regulates mcl-1 in human myeloma cells through JAK/STAT rather than ras/MAP kinase pathway. Eur J Immunol 29(12):3945–3950
35. Kawano M, Hirano T, Matsuda T, Taga T, Horii Y, Iwato K et al (1988) Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. Nature 332:83–85
36. Uchiyama H, Barut BA, Mohrbacher AF, Chauhan D, Anderson KC (1993) Adhesion of human myeloma-derived cell lines to bone marrow stromal cells stimulates interleukin-6 secretion. Blood 82(12):3712–3720
37. Szczepek AJ, Belch AR, Pilarski LM (2001) Expression of IL-6 and IL-6 receptors by circulating clonotypic B cells in multiple myeloma: potential for autocrine and paracrine networks. Exp Hematol 29(9):1076–1081
38. Cheung W-C, Van Ness B (2002) Distinct IL-6 signal transduction leads to growth arrest and death in B cells or growth promotion and cell survival in myeloma cells. Leukemia 16:1182–1188
39. Frank DA, Mahajan S, Ritz J (1998) Activation of T cells through CD2 leads to the delayed and prolonged activation of STAT1. Blood 92:701a
40. Chauhan D, Uchiyama H, Akbarali Y, Urashima M, Yamamoto K, Libermann TA et al (1996) Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-kappa B. Blood 87:1104–1112
41. Alas S, Bonavida B (2003) Inhibition of constitutive STAT3 activity sensitizes resistant non-Hodgkin's lymphoma and multiple myeloma to chemotherapeutic drug-mediated apoptosis. Clin Cancer Res 9:316–326
42. De Vos J, Jourdan M, Tarte K, Jasmin C, Klein B (2000) JAK2 tyrosine kinase inhibitor tyrphostin AG490 downregulates the mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT) pathways and induces apoptosis in myeloma cells. Br J Haematol 109(4):823–828
43. Oshiro MM, Landowski TH, Catlett-Falcone R, Hazlehurst LA, Huang M, Jove R et al (2001) Inhibition of JAK kinase activity enhances Fas-mediated apoptosis but reduces cytotoxic activity of topoisomerase II inhibitors in U266 myeloma cells. Clin Cancer Res 7(12):4262–4271
44. Chim CS, Fung TK, Cheung WC, Liang R, Kwong YL (2004) SOCS1 and SHP1 hypermethylation in multiple myeloma: implications for epigenetic activation of the Jak/STAT pathway. Blood 103(12):4630–4635
45. Galm O, Yoshikawa H, Esteller M, Osieka R, Herman JG (2003) SOCS-1, a negative regulator of cytokine signaling, is frequently silenced by methylation in multiple myeloma. Blood 101:2784–2788
46. Bharti AC, Shishodia S, Reuben JM, Weber D, Alexanian R, Raj-Vadhan S et al (2004) Nuclear factor–kB and STAT3 are constitutively active in CD138+ cells derived from multiple myeloma patients, and suppression of these transcription factors leads to apoptosis. Blood 103:3175–3184
47. Brocke-Heidrich K, Kretzschmar AK, Pfeifer G, Henze C, Loffler D, Koczan D et al (2004) Interleukin-6-dependent gene expression profiles in multiple myeloma INA-6 cells reveal a Bcl-2 family-independent survival pathway closely associated with Stat3 activation. Blood 103(1):242–251

48. Chauhan D, Kharbanda S, Ogata A, Urashima M, Teoh G, Robertson M et al (1997) Interleukin-6 inhibits Fas-induced apoptosis and stress-activated protein kinase activation in multiple myeloma cells. Blood 89:227–234
49. Nelson EA, Walker SR, Li W, Liu XS, Frank DA (2006) Identification of human STAT5-dependent gene regulatory elements based on interspecies homology. J Biol Chem 281(36):26216–26224
50. Vallania F, Schiavone D, Dewilde S, Pupo E, Garbay S, Calogero R et al (2009) Genome-wide discovery of functional transcription factor binding sites by comparative genomics: the case of Stat3. Proc Natl Acad Sci USA 106(12):5117–5122
51. Oh YM, Kim JK, Choi Y, Choi S, Yoo JY (2009) Prediction and experimental validation of novel STAT3 target genes in human cancer cells. PLoS One 4(9):e6911
52. Robertson G, Hirst M, Bainbridge M, Bilenky M, Zhao Y, Zeng T et al (2007) Genome-wide profiles of STAT1 DNA association using chromatin immunoprecipitation and massively parallel sequencing. Nat Methods 4(8):651–657
53. Nelson EA, Walker SR, Alvarez JA, Frank DA (2004) Isolation of unique STAT5 targets by chromatin immunoprecipitation-based gene identification. J Biol Chem 279:54724–54730
54. Akgul C (2009) Mcl-1 is a potential therapeutic target in multiple types of cancer. Cell Mol Life Sci 66(8):1326–1336
55. Isomoto H, Kobayashi S, Werneburg NW, Bronk SF, Guicciardi ME, Frank DA et al (2005) Interleukin 6 upregulates myeloid cell leukemia-1 expression by a JAK/STAT3 pathway in cholangiocarcinoma cells. Hepatology 42:1329–13338
56. Sharma SV, Settleman J (2007) Oncogene addiction: setting the stage for molecularly targeted cancer therapy. Genes Dev 21(24):3214–3231
57. Chonghaile TN, Letai A (2009) Mimicking the BH3 domain to kill cancer cells. Oncogene 27(S1):S149–S157
58. Le Gouill S, Podar K, Amiot M, Hideshima T, Chauhan D, Ishitsuka K et al (2004) VEGF induces Mcl-1 up-regulation and protects multiple myeloma cells against apoptosis. Blood 104(9):2886–2892
59. Podar K, Anderson KC (2007) Inhibition of VEGF signaling pathways in multiple myeloma and other malignancies. Cell Cycle 6(5):538–542
60. Podar K, Richardson PG, Chauhan D, Anderson KC (2007) Targeting the vascular endothelial growth factor pathway in the treatment of multiple myeloma. Expert Rev Anticancer Ther 7(4):551–566
61. Medinger M, Fischer N, Tzankov A (2010) Vascular endothelial growth factor-related pathways in hemato-lymphoid malignancies. J Oncol 2010:729725
62. Viatour P, Dejardin E, Warnier M, Lair F, Claudio E, Bureau F et al (2004) GSK3-Mediated BCL-3 Phosphorylation Modulates Its Degradation and Its Oncogenicity. Mol Cell 16(1):35–45
63. Brenne AT, Fagerli UM, Shaughnessy JD Jr, Vatsveen TK, Ro TB, Hella H et al (2009) High expression of BCL3 in human myeloma cells is associated with increased proliferation and inferior prognosis. Eur J Haematol 82(5):354–363
64. Brocke-Heidrich K, Ge B, Cvijic H, Pfeifer G, Loffler D, Henze C et al (2006) BCL3 is induced by IL-6 via Stat3 binding to intronic enhancer HS4 and represses its own transcription. Oncogene 25(55):7297–7304
65. Fujita T, Nolan GP, Liou HC, Scott ML, Baltimore D (1993) The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-kappa B p50 homodimers. Genes Dev 7(7b):1354–1363
66. Kuwata H, Watanabe Y, Miyoshi H, Yamamoto M, Kaisho T, Takeda K et al (2003) IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF-{alpha} production in macrophages. Blood 102(12):4123–4129
67. Tsuyama N, Danjoh I, Otsuyama K, Obata M, Tahara H, Ohta T et al (2005) IL-6-induced Bcl6 variant 2 supports IL-6-dependent myeloma cell proliferation and survival through STAT3. Biochem Biophys Res Commun 337:201–208

68. Hideshima T, Mitsiades C, Ikeda H, Chauhan D, Raje N, Gorgun G et al (2010) A proto-oncogene BCL6 is up-regulated in the bone marrow microenvironment in multiple myeloma cells. Blood 115(18):3772–3775
69. Walker SR, Nelson EA, Zou L, Chaudhury M, Signoretti S, Richardson A et al (2009) Reciprocal effects of STAT5 and STAT3 in breast cancer. Mol Cancer Res 7(6):966–976
70. Papagiannakopoulos T, Shapiro A, Kosik KS (2008) MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. Cancer Res 68(19):8164–8172
71. Pichiorri F, Suh SS, Ladetto M, Kuehl M, Palumbo T, Drandi D et al (2008) MicroRNAs regulate critical genes associated with multiple myeloma pathogenesis. Proc Natl Acad Sci USA 105(35):12885–12890
72. Wernig G, Kharas MG, Okabe R, Moore SA, Leeman DS, Cullen DE et al (2008) Efficacy of TG101348, a selective JAK2 inhibitor, in treatment of a murine model of JAK2V617F-induced polycythemia vera. Cancer Cell 13(4):311–320
73. Li J, Favata M, Kelley JA, Caulder E, Thomas B, Wen X et al (2010) INCB16562, a JAK1/2 selective inhibitor, is efficacious against multiple myeloma cells and reverses the protective effects of cytokine and stromal cell support. Neoplasia 12(1):28–38
74. Burger R, Le Gouill S, Tai YT, Shringarpure R, Tassone P, Neri P et al (2009) Janus kinase inhibitor INCB20 has antiproliferative and apoptotic effects on human myeloma cells in vitro and in vivo. Mol Cancer Ther 8(1):26–35
75. Nelson EAHT, Gashin L, Walker SR, Lynch RA, Chauhan D, Anderson KC, Frank DA (2006) Nifuroxazide inhibits STAT3 function and shows potent anti-tumor activity against multiple myeloma. Blood 108:3450
76. Kim HY, Park EJ, Joe EH, Jou I (2003) Curcumin suppresses Janus kinase-STAT inflammatory signaling through activation of Src homology 2 domain-containing tyrosine phosphatase 2 in brain microglia. J Immunol 171:6072–6079
77. Lin L, Deangelis S, Foust E, Fuchs J, Li C, Li PK et al (2010) A novel small molecule inhibits STAT3 phosphorylation and DNA binding activity and exhibits potent growth suppressive activity in human cancer cells. Mol Cancer 9:217
78. Stasi R, Brunetti M, Parma A, Di Giulio C, Terzoli E, Pagano A (1998) The prognostic value of soluble interleukin-6 receptor in patients with multiple myeloma. Cancer 82(10):1860–1866
79. Bataille R, Barlogie B, Lu ZY, Rossi JF, Lavabre-Bertrand T, Beck T et al (1995) Biologic effects of anti-interleukin-6 murine monoclonal antibody in advanced multiple myeloma. Blood 86(2):685–691
80. Fulciniti M, Hideshima T, Vermot-Desroches C, Pozzi S, Nanjappa P, Shen Z et al (2009) A high-affinity fully human anti-IL-6 mAb, 1339, for the treatment of multiple myeloma. Clin Cancer Res 15(23):7144–7152
81. Hausherr A, Tavares R, Schaffer M, Obermeier A, Miksch C, Mitina O et al (2007) Inhibition of IL-6-dependent growth of myeloma cells by an acidic peptide repressing the gp130-mediated activation of Src family kinases. Oncogene 26(34):4987–4998
82. Todoerti K, Barbui V, Pedrini O, Lionetti M, Fossati G, Mascagni P et al (2010) Pleiotropic anti-myeloma activity of ITF2357: inhibition of interleukin-6 receptor signaling and repression of miR-19a and miR-19b. Haematologica 95(2):260–269
83. Yoshikawa H, Matsubara K, Qian GS, Jackson P, Groopman JD, Manning JE et al (2001) SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. Nat Genet 28:29–35
84. Yamamoto M, Nishimoto N, Davydova J, Kishimoto T, Curiel DT (2006) Suppressor of cytokine signaling-1 expression by infectivity-enhanced adenoviral vector inhibits IL-6-dependent proliferation of multiple myeloma cells. Cancer Gene Ther 13(2):194–202
85. Depil S, Saudemont A, Quesnel B (2003) SOCS-1 gene methylation is frequent but does not appear to have prognostic value in patients with multiple myeloma. Leukemia 17(8):1678–1679
86. Gorgun G, Calabrese E, Soydan E, Hideshima T, Perrone G, Bandi M et al (2010) Immunomodulatory effects of lenalidomide and pomalidomide on interaction of tumor and bone marrow accessory cells in multiple myeloma. Blood 116(17):3227–3237

87. Wang T, Niu G, Kortylewski M, Burdelya L, Shain K, Zhang S et al (2004) Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. Nat Med 10(1):48–54
88. Ihle JN (1995) The Janus protein tyrosine kinase family and its role in cytokine signaling. Adv Immunol 60:1–35
89. Lin TS, Mahajan S, Frank DA (2000) STAT signaling in the pathogenesis and treatment of leukemias. Oncogene 19:2496–2504
90. Frank DA (1999) STAT signaling in the pathogenesis and treatment of cancer. Mol Med 5:432–456
91. Turkson J, Ryan D, Kim JS, Zhang Y, Chen Z, Haura E et al (2001) Phosphotyrosyl peptides block Stat3-mediated DNA binding activity, gene regulation, and cell transformation. J Biol Chem 276(48):45443–45455
92. Turkson J, Kim JS, Zhang S, Yuan J, Huang M, Glenn M et al (2004) Novel peptidomimetic inhibitors of signal transducer and activator of transcription 3 dimerization and biological activity. Mol Cancer Ther 3:261–269
93. Xi S, Gooding WE, Grandis JR (2005) In vivo antitumor efficacy of STAT3 blockade using a transcription factor decoy approach: implications for cancer therapy. Oncogene 24(6):970–979
94. Chan KS, Sano S, Kiguchi K, Anders J, Komazawa N, Takeda J et al (2004) Disruption of Stat3 reveals a critical role in both the initiation and the promotion stages of epithelial carcinogenesis. J Clin Invest 114(5):720–728
95. Wang LH, Yang XY, Kirken RA, Resau JH, Farrar WL (2000) Targeted disruption of Stat6 DNA binding activity by an oligonucleotide decoy blocks IL-4-driven TH2 cell response. Blood 95:1249–1257
96. Trauger JW, Baird EE, Dervan PB (1996) Recognition of DNA by designed ligands at subnanomolar concentrations. Nature 382:559–561

Part II
Myeloma Microenvironment

Chapter 8
The Bone Marrow Microenvironment: Novel Targets to Circumvent Minimal Residual Disease and Drug Resistance in Multiple Myeloma

Kenneth H. Shain and William S. Dalton

Abstract The bone marrow micro environment has long been appreciated to support multiple myeloma cell pathogenesis. It is evident that this niche may be as important a therapeutic target as the malignant myeloma cells. This stems from research over the last one to two decades demonstrating that determinants of the bone marrow milieu are integral in myeloma pathogenesis, survival, immune surveillance, and resistance to both traditional and novel therapeutic agents. Early studies elucidating drug resistance in this disease focused on the myeloma cells and found that genetic, acquired changes, in the expression or function of specific gene products mediated cell survival. Subsequently, dynamic, de novo mechanisms coordinated by the tumor microenvironment have been shown to confer an environmental mediated- drug resistance (EM-DR). Appreciation of EM-DR has spawned an exciting path of preclinical and clinical research focused on attenuating the pro-myeloma aspects of the tumor microenvironment. Within this chapter we will provide an overview of the bone marrow microenvironment in the context of multiple myeloma and how these determinants contribute to minimal residual disease and subsequent treatment failure. With increased understanding of the bone marrow niche and EM-DR, numerous novel therapies are under development targeting the microenvironment with the anticipation of improved clinical outcomes.

K.H. Shain, M.D., Ph.D
Experimental Therapeutics Program,
H. Lee Moffitt Cancer Center and Research Institute,
12902 Magnolia Drive, Tampa, FL 33612, USA
e-mail: ken.shain@moffitt.org

W.S. Dalton, Ph.D., M.D. (✉)
Experimental Therapeutics Program,
H. Lee Moffitt Cancer Center and Research Institute,
12902 Magnolia Drive, Tampa, FL 33612, USA

Departments of Experimental Therapeutics and Oncologic Sciences,
H. Lee Moffitt Cancer Center and Research Institute, University of South Florida,
12902 Magnolia Drive, Tampa, FL 33612, USA
e-mail: william.dalton@moffitt.org

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_8,

8.1 Introduction

Multiple myeloma (MM) is a disease of bone marrow (BM)-resident malignant plasma cells. As a consequence of multidrug resistance (MDR), this plasma dyscrasia is plagued by therapy failure and the ultimate demise of the patient. This aspect of myeloma has fostered a great deal of research into delineating the mechanisms by which MM cells evade regimens of chemotherapy. Recent clinical trials incorporating the novel compounds thalidomide, lenalidomide, and bortezomib have provided a rationale for overcoming drug resistance [1–3]. The clinical success of these agents is attributable to both direct cytotoxicity and negative regulation of pro-myeloma determinants of the microenvironment. Unfortunately, even with the clinical gains made from these agents, resistance to these compounds remains inevitable; as although highly effective, disease relapse remains unavoidable. Therefore, continued investigation into the mechanisms of both intrinsic and environmental drug resistance remains paramount for continued gains in the control, if not cure, of MM.

An important concept in myeloma treatment failure is that of minimal residual disease (MRD) and its contribution to disease relapse and drug resistance. Not exclusive to MM, MRD represents limitations in our technology to detect *small* populations (*a billion cells*) of disease as well as limitations in our therapeutic armamentarium. With current therapy and technology, complete remission (CR) is defined by the International Myeloma Working Group as the absence of detectable serum and urine monoclonal proteins by immunofixation as well as an undetectable plasma cell population within the BM [4]. Currently, investigational techniques using patient-specific polymerase chain reaction (PCR) and multiparameter flow cytometry (MFC) are able to detect clonal plasma cell populations in patients meeting the IMWG definition of CR [5–8]. The enduring population of cells represents MRD or cells that have *evaded* therapy. Subsequent expansion of these residual cells correlates with disease relapse [7]—frequently with an MDR phenotype making salvage therapy less successful even with the utilization of novel therapeutic agents. Moreover, the incredible benefit bestowed by high-dose chemotherapy and autologous stem cell transplant, in the appropriate population, is predicated on the concept that a greater reduction in MRD equates to prolonged progression-free survival (PFS) and overall survival (OS) [9–11]. This concept has been taken a step further within the integration of novel and traditional therapies with tandem transplant [9–12]. The clinical risk versus benefit of chemotherapy (*novel* agent-based), single versus tandem transplant, remains controversial [13], as does the relationship between residual disease and clinical outcome. However, multiple studies have indicated that the degree of response (CR, VGPR, PR) correlates with an improvement in EFS and OS regardless of the therapeutic modality. Therefore, targeting MRD should be a therapeutic goal in the appropriate patient population [14]. The significance of the residual population is further highlighted by a recent report by Paiva et al. examining the use of a “more sensitive” assessment of MRD, MFC, in patients achieving at least a CR with novel agent therapy [15]. The authors demonstrated that patients with MFC-positive MRD [CR and

stringent CR (SFLC-negative CR)] had a poorer PFS and TTP when compared to patients without (i.e., MFC-negative), termed immonophenotypic response (IR) [15]. The overall survival data have yet to be reported and will have a considerable bearing on the significance of MRD in myeloma. To this end, it is apparent that continued investigation and understanding of the determinants modulating EM-DR are integral in the design of novel therapies and greater success in the control, if not eradication, of MM via a reduction in MRD.

8.2 Genetic and Environmental Contributions to Myeloma

Myeloma results from a sequence of genetic alterations within a clone that facilitates an escape from the programmed constraints governing proliferation and death. In MM, it has been proposed that these requirements are at least partially fulfilled by the nonrandom translocation of a gene(s) that normally functions as determinants of cell proliferation or cell survival to regions juxtaposed to active IgH enhancer elements located on the long arm of chromosome 14 (less frequently light chain enhancers) that occur secondary to errors in IgH switch recombination. These genetic alterations, in turn, facilitate the overexpression and activity of an oncogene (primarily MMSET, FGFR3, CCDN3, CCDN1, c-MAF, and MAFB) driving transformation. Subsequent secondary IgH translocations with c-myc and activating mutations in Ras (NRAS and KRAS), FGFR3, and TRAF3 are acquired throughout the course of disease and correlate with progression or relapse [16]. Loss of entire chromosomes (hypodiploidy) or a portion of chromosomes contribute to myelomagenesis exemplified by the high-risk stratification of patients with deletion of the short arm of chromosome 13 (del13) by cytogenetics or with deletion of a portion of chromosome 17 (17p13), potentially representing losses of the tumor suppressors Rb and p53, respectively.

Within the backdrop of a genetic disease, it is appreciated that *both* genetic alterations and microenvironmental effectors contribute to the deregulation of life and death of MM cells [16]. The BM niche provides a sanctuary to resident MM cells via both soluble and physical components [interactions between MM cells and specific extracellular matrix (ECM) components or other cellular components, e.g., BM stromal cells (BMSCs)]. Soluble and physical determinants of the BM are central in homing to and survival within the BM microenvironment [1, 17, 18]. Although discussed and examined as separate entities, resistance to therapy likely involves the "collaboration" between dynamic, de novo resistance modulated by the BM microenvironment and heritable, acquired mechanisms of drug resistance selected by drug exposure. The former providing an initial protective effect and contributing to MRD, and the latter selected under chronic exposure to therapy ultimately resulting in the expansion of a MDR population (relapse) (Fig. 8.1). Based on this hypothesis, elucidation of the environmental determinants that afford a protective advantage to resident myeloma cells, we may be able to overcome de novo and acquired MDR. Within this chapter, we will discuss the important aspects of the BM microenvironment that contribute to EM-DR.

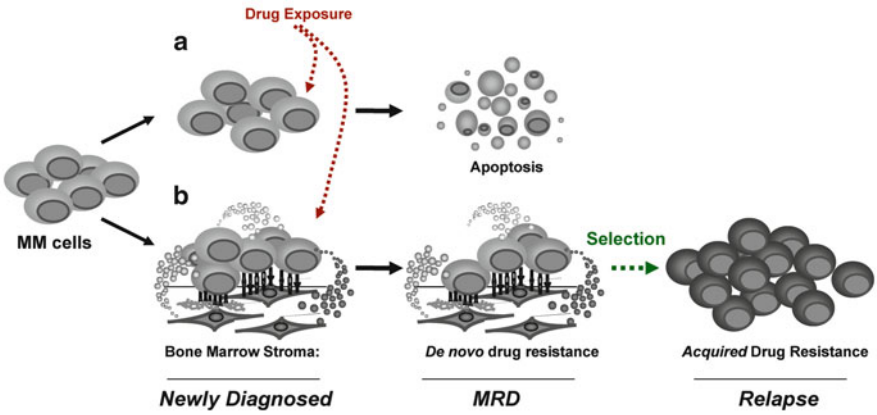


Fig. 8.1 *A proposed model for the minimal residual disease and the subsequent development of selected MDR*. The mechanisms of drug resistance can be divided into two categories: genetic or *acquired* mechanisms and environmental or de novo mechanisms of MDR (EM-DR). It has been proposed that therapy resistance in MM involves a sequential process involving de novo resistance and acquired mechanisms of drug resistance. In this model, the bone marrow niche affords an initial pro-survival environment. To this end, cells interacting with the key determinants are protected at the expense of other cells. The resultant population represents MRD. Within this sanctuary, following exposure to therapeutic (and/or other genotypic) stressors, selection of mechanisms of acquired MDR facilitates the expansion of an MDR population of MM cells (relapse). If this hypothesis is correct, then targeting the bone marrow microenvironment will increase therapeutic efficiency and lead to better control, if not cure of this mortal disease

8.3 The Bone Marrow Niche

Normal plasma cell development culminates in the homing of post-germinal center (GC), long-lived, plasma cells to survival niches within the BM microenvironment [19–21]. Plasma cells are terminally differentiated, antibody-producing B lymphocytes that have undergone class-switch recombination, somatic hypermutation. Maturation of these cells has involved migration from the BM, to secondary lymphoid organs (spleen or lymph nodes), and back to the BM (arguments can be made that the BM is also a secondary lymphoid organ and some plasma may not leave the marrow). Post-GC BM homing correlates with the expression of BLIMP (B-cell lymphocyte-induced maturation protein)-1 in secondary lymphoid organs modulating the repression of CXCR5 (CXC-chemokine receptor 5) and the expression of CXCR4 and α4-integrins [20, 22, 23]. CXCR4 modulates migration to CXCL12 [CXC-ligand-12/stromal cell-derived factor (SDF)-1]-rich regions of BM niche rich and expression α4 integrins [19, 24, 25]. In turn, α4-containing integrin heterodimers facilitate adhesion to VCAM (vascular cell adhesion molecule)-1 as well as other homo- and heterotypic adhesion molecules. Within the BM, plasma cell adherence to BMSCs and likely other juxtaposed cells leads to the production of IL (interleukin)-6, BAFF (B-cell-activating factor), bFGF (basic-fibroblast growth factor), and other soluble factors that provide crucial survival factors to these long-lived antibody-producing cells concordant with BM homeostasis.

It is not difficult to see how the veritable cornucopia of pro-survival and proliferative effectors within the BM microenvironment would also benefit the progression of a malignancy, if not its evolution. MM is a malignancy of clonal antibody producing (save for rare cases) post-GC (or marginal zone) plasma cells facilitated by errors in class-switch recombination and/or somatic hypermutation [26, 27]. Like their normal counterparts, these mature B cells have migrated to the BM via various chemo-attractants and adhesion molecules [24, 25]. Unlike their normal counterparts, myeloma cells not only benefit from the normal effectors within the BM niche but also *hijack* and *contribute* to the environment in a manner that promotes tumorigenesis, altered bone metabolism, neovascularization, and drug resistance [28–30]. Myeloma cell adhesion induces BM stromal cell secretion of chemokines, cytokines, and growth factors [3, 27, 28, 31]. IL-6 remains one of the most prominent myeloma growth factors; however, a growing list of soluble effectors are also induced by myeloma cell–stromal cell interactions. These factors include cytokines: IL-1β, IL-3, IL-15, IL-21, TNF-α, OSM, LIF; chemokines: IL-8, CXCL12, MIP1-α; growth factors: VEGF (vascular endothelial growth factor), FGF (family), IGF-1 (insulin-like GF-1), HGF (hepatocyte GF); and other pro-myeloma factors: Dkk-1 (Dickkopf-1), RANKL (receptor activator of nuclear factor kappaB (NF-κB) ligand), BAFF (B-cell activation growth factor), Wnt (family), and TGF-β (transforming GF), among others [27, 32, 33]. In addition to direct proliferative and survival effects, soluble factors also impart the adhesion of myeloma cells to adjacent cellular components (stromal cells, dendritic cells, mesenchymal stem cells (MSCs), macrophages, and osteoclasts) and extracellular matrices [fibronectin (FN), collagens, vitronectin, glucose-aminoglycans (GAG), hyaluronan, and laminins] via a host of adhesion molecules. The most prominent being VLA-4 (α4/β1/CD49d/CD29), VLA-5 (α5/β1/CD49e/CD29), CD44, VCAM-1, intracellular CAM (ICAM) (CD54), NCAM (CD56), CD74, and CS-1 (CD2-subset-1) among others [3, 27, 31]. Extracellular stimulation by soluble factors or direct adhesion facilitates networks of intracellular cascades. For most of the soluble and physical determinants listed above, a number of signaling cascades have been delineated that translated these extracellular stimuli to biology function. Importantly, these pathways are typically investigated and discussed in a vacuum; however, it is evident that collaborative signaling alters that biologic outcome [34–36]. To this end, it is important to account the network of effectors within the myeloma microenvironment when attempting to identify the most relevant targetable pathways [37].

Within the scope of this book chapter, we will discuss the manner in which a number of these BM determinants and subsequent signaling cascades confer drug resistance to resident myeloma cells. However, it is important to remark that these malignant cells have additional and likely interrelated bearing on bone metabolism and angiogenesis within the BM niche. Osteolytic disease is one of the more significant hallmarks of MM occurring through alterations in osteoclast to osteoblast ratios and subsequent increase in focal bone catabolism [38]. This is too mediated by the altered signaling between soluble and adherent stimuli between myeloma cells and osteoclasts, dendritic cells, activated T cells, and BMSCs [38].

Myeloma cell production of RANKL, TGF-β, HGF, IL-3, and Dkk-1 inhibit the growth of osteoblasts. Simultaneously, RANKL, SDF-1, MIP1-α, and TNF-α stimulate bone resorption through the positive regulation of osteoclasts [30, 39]. Together, these effects result in the net resorption of bone in focal regions—lytic bone lesions. Focal lesions are sites of a number of heterotypic adhesive, paracrine, autocrine signaling between myeloma cells and adjacent cellularity. These lesions are likely relevant in drug resistance as these focal regions are potential sites for MRD. Evidence of this is suggested in studies demonstrating decreased EFS and OS in patients in CR with identifiable focal lesions on whole body-MRI or PET-CTs relative to patients with negative imaging [40, 41].

A number of studies have implicated that increased microvessel density (MVD) correlates with disease state, suggesting that increased BM angiogenesis is important to myeloma progression [42, 43]. This theorem originates from observations of the increased expression of proangiogenic factors including, VEGF, bFGF, angiopoietin (ang)-1, and ang-2 myeloma patients [43]. The most compelling evidence stems from the examination of BM MVD that revealed a greater density in patients with active disease relative to patients with MGUS [42, 43]. Du et al. demonstrated that MVD increased from normal BM samples (normal stem cell donors) to MGUS to active disease [43]. The authors further demonstrated that treatment with thalidomide reduced MVD in three of seven patients. Other compelling evidence was elucidated from studies demonstrating that elevated MVD was a univariant predicted poorer overall survival in myeloma patients [44]. Lastly, it is interesting to note that studies characterizing the *MM cancer stem cell* have suggested that two lineages are populated by these progenitor cells: (1) the prototypical *myeloma plasma cell* and (2) a *myeloma monocytoid cell* [29]. With the use of patient-specific FISH, further investigation demonstrated that the monocytoid lineage populated a *myeloma vascular endothelial cell* lineage [29]. The authors suggest that not only do myeloma cells produce factors that promote angiogenesis, but may, in fact, also directly contribute to increased MVD, nutrient delivery, and plasma cell homing to the marrow. Together, these data demonstrate the incredible influence the BM microenvironment may have on myeloma pathobiology outside the context of MDR.

8.4 The Bone Marrow Niche and Drug Resistance

The unique dependence of myeloma on its niche has made this neoplasm a model for defining the influence of the tumor microenvironment network on the disease. The cytokines, chemokines, growth factors, and adhesive matrices of the BM microenvironment facilitate MM cell homing and expansion [3, 17, 45–47]. The biological mechanisms regulating MM cell homing to the BM involve the regulation of soluble and physical determinants of the microenvironment [17]. In turn, resident myeloma cells are afforded sanctuary from host immune surveillance as well as therapeutic agents. Therefore, elucidation of the mechanisms modulating homing of MM to the BM and de novo drug resistance will facilitate the rational

development of novel pharmacological agents and/or regimens in MM by targeting MRD. Appreciation of this concept has spawned an arm of preclinical and clinical studies with the sole target of the BM microenvironment.

8.5 Soluble Factors

Early observations of increased levels of cytokines and chemokines within the BM microenvironment of MM patients have long suggested a contribution of IL-6 and other soluble factors in MM pathogenesis. A number of these soluble factors, including interleukin (IL)-6, insulin-like growth factor (IGF)-1, interferon (IFN)-α, and fibroblast growth factor (FGF)-3, have subsequently been shown to confer resistance to cytotoxic insult (Table 8.1) [3, 48–50]. Other soluble factors are paramount in the homing to and maintenance of adherent myeloma cells within the BM. The chemokine SDF-1/CXCL12 is a critical regulator of myeloma–BM niche via binding to CXC chemokine receptor (CXCR)-4/CD184 and the more recently identified CXCR7 [17, 51, 52]. Signaling following CXCR4 (or CXCR7) ligation has been shown to activate Janus kinase (JAK)/STAT signaling, but primarily involves heterotrimeric G-proteins (Gα/Gβ/Gγ) [53]. Ligand–receptor binding facilitates the release of the trimeric protein and activation of phosphatidylinositol (PI) 3-kinase, Rac, Rho, Ras/Raf, and phospholipase C [24, 53, 54]. The SDF-1/CXCL12 and CXCR4 axis modulates adhesion and chemotaxis interactions with VCAM-1 and FN [55, 56]. SDF-1 has additionally been demonstrated to promote cell survival [57].

Of the soluble determinants of the BM microenvironment, IL-6 remains the prototypical growth and survival factor in myeloma. Moreover, numerous additional factors contribute to myeloma by either directly or indirectly promoting the production/secretion of IL-6 by BMSCs, MSCs, osteoclasts, and malignant cells. TNF-α, IL-1β, FGF-3, TGF-β, and VEGF have been shown to modulate IL-6 expression in the context of the BM niche [3]. These results highlight the prominent role of IL-6 in MM and propagated research characterizing the biology of IL-6 signaling. As such, IL-6 will be used as an example of soluble factor signaling. Signal transduction follows binding of IL-6 to gp80/IL-6Ralpha and recruitment of gp130/CD130 [58]. The induced dimerization of gp130 results in receptor phosphorylation by constitutively bound JAK family tyrosine kinases (JAK1, JAK2, and Tyk2) [59]. IL-6 binding and receptor multimerization facilitates the phosphorylation of gp130 and initiation of three major signaling pathways in MM cells: (1) the Ras/Raf–mitogen-activated protein kinase kinase–extracellular signal-related kinase (ERK)1/2 pathway [60]; (2) the PI3-kinase pathway; (3) the JAK/signal transducer and activator of transcription pathway (Fig. 8.3a) [61]. Src-family tyrosine kinases (SFKs) have also been identified as mediators of IL-6 signaling events [1, 62, 63]. Whether these kinases (SFKs) act to enhance STAT3, PI3-kinase, and Ras signaling or represent an independent pathway has yet to be completely determined. SFKs-dependent enhancement of STAT3 activation and proliferative signaling following IL-6 cross-linking has been observed [63].

Table 8.1 Environment-mediated drug resistance (EM-DR) in MM

Determinants of the myeloma bone marrow niche
Soluble factors
Cytokines:
Interleukin (IL)-6
IL-1β
IL-3
IL-10
IL-15
IL-21
Tumor necrosis factor (TNF)-α
OSM (oncostatin M)
BAFF (B-cell activation growth factor)
A proliferation-induced ligand (APRIL)
Chemokines:
IL-8
CXCL12
MIP1-α
Growth factors:
Insulin-like growth factor (IGF)
Vascular endothelial growth factor (VEGF)
Fibroblast growth factor (FGF Family)
Hepatocyte growth factor (HGF)
Other:
Interferon α (IFN)
Dkk-1 (Dickkopf-1)
Receptor activator of nuclear factor kappaB (NF-κB) ligand (RANKL)
Wnt (family)
Transforming growth factor (TGF)-β
Direct cell contact
Cell adhesion molecules:
VLA-4 (α4/β1/CD49d/CD29)
VLA-5 (α5/β1/CD49e/CD29)
CD44
VCAM-1 (CD106)
ICAM (CD54)
NCAM (CD56)
CD74
CS-1 (CD2-subset-1)
Notch
Cellular components:
Bone marrow stromal cells (BMSC)
Mesenchymal stem cells (MSC)
Bone marrow dendritic cells
Vascular endothelial cells
Immune effectors (activated T lymphocytes)
Osteoclasts

(continued)

Table 8.1 (continued)

Determinants of the myeloma bone marrow niche
Osteoblasts
Macrophages
Multiple myeloma monocytoid cells
Multiple myeloma vascular endothelial cells

This table represents a sampling of the determinants and is not all encompassing

Each of these signal transduction pathways has been implicated in IL-6-mediated resistance to both physiological and chemotherapy-mediated apoptosis. The variety of downstream targets demonstrates that a single soluble factor has the potential to regulate drug sensitivity through multiple mechanisms. For the purposes of this review, IL-6 serves as a representative signaling soluble environmental determinant of EM-DR. However, the lack of definitive clinical benefits of IL-6 antagonism suggests that a significant degree of redundancy exists within the microenvironment. As stated, numerous signaling soluble determinants have been shown to confer resistance to cytotoxic stress through similar signaling effectors independently of IL-6 [48–50]. To this end, targeting of a single effector may not be sufficient. Simultaneous targeting complementary signaling pathways will likely be necessary for improved clinical efficacy.

8.5.1 Ras/Raf/MEK/ERK1/2 Signaling

IL-6 signaling primarily involves activation of three signaling cascades: Ras/Raf–MEK–ERK1/2, PI3-kinase–Akt, and JAK–STAT. Each of these pathways has been shown to modulate cellular growth and survival. The oncogenic role of the Ras/Raf–MEK–ERK1/2 pathway has long been established especially in the context of mutant Ras. This pathway has also been linked to proliferation and survival in response to cytokines, growth factors, chemokines, and adhesive matrices [64, 65]. The pro-apoptotic Bcl-2 family member Bim represents a putative downstream target of this signaling pathway [66]. The abrogation of MEK1/2 activity correlated with increased phosphorylation/downregulation of Bim_{EL} and alterations in mitochondrial integrity. Whether directly related to Bim, MEK–ERK1/2 signaling has been shown to be integral in the survival signaling modulated by IL-6 and BMSCs [64]. Moreover, inhibition of this pathway sensitized BMSC-resident myeloma cells to both novel and traditional therapeutic agents [64, 65]. This may be in part related to MEK-dependent regulation of the paracrine factors BAFF, APRIL, and MIP-1α by osteoclasts [39, 64, 65]. More recently, MEK has been elucidated as a key regulator of the key oncogene c-MAF, which has significance in the context of both environmental and heritable myeloma disease progression [65, 67, 68]. Collectively, these data indicate that Ras/Raf–MEK–ERK1/2 signaling remains an exciting therapeutic target.

8.5.2 PI3-Kinase/Akt Signaling

The different effectors of IL-6-mediated drug resistance appear to have different (and in some cases specific) anti-apoptotic mechanisms. The PI3-kinase pathway signaling following IL-6 stimulation was demonstrated to confer resistance to dexamethasone (dex), but not ionizing radiation (IR) or CD95/Fas/Apo-1-induced apoptosis. Dex resistance involves a PI3-kinase-dependent dephosphorylation of focal adhesion tyrosine kinase (RAFTK/Pyk2/CAKβ) by SH2-containing protein tyrosine phosphatase (SHP) [69]. Further, p53, NF-κB, Bad, Survivin, XIAP, and procaspase-9 are also putative downstream effectors of PI3-kinase and Akt in MM and other cell systems [70–72]. Molecular attenuation of PI3-kinase (p110δ) with siRNA has been shown to specifically control myeloma proliferation and survival in the context of BMSCs and in in vivo SCID-hu murine models [73, 74]. Importantly, the authors demonstrated that this was specific for myeloma cells, sparing the normal hematopoietic cells [73]. These results suggest that by targeting PI3-kinase, we can overcome EM-DR and maintain a high therapeutic window.

PI3-kinase/Akt activation has been linked to MM cell survival with putative downstream targets including the serine/threonine kinase mTOR (mammalian targets of rapamycin). mTOR is an exciting target of novel agents in many tumor types including myeloma [75, 76]. The activity of mTOR is proposed to involve two signaling protein complexes: TORC1 and TORC2 [27, 72, 77]. It has been proposed that TORC1 mediates the proliferative aspects of Akt signaling following receptor tyrosine kinase stimulation. TORC1 also functions in a negative feedback loop, controlling the dephosphorylation of Akt [77]. TORC2, in contrast, may be involved in the anti-apoptotic Akt signaling and is known to phosphorylate Akt [77]. Specific and dual mTOR complex inhibitors are under active development and testing in myeloma due to the potential anti-microenvironment and/or anti-myeloma effects [78–80].

8.5.3 JAK/STAT Signaling

The role of JAK/STAT signaling is not limited to neoplastic transformation of MM. The identification of mutant JAK2 in other hematologic malignancies including myeloproliferative neoplasms (MPNs), acute myelogenous leukemias (AML), and acute lymphoblastic leukemias (ALL) has facilitated an increased interest in this signaling partnership as a target for therapy [81]. IL-6 ligation of cognate receptor facilitates the autophosphorylation and transphosphorylation of JAK constitutively associated with gp130, facilitating recruitment and activation of STAT3. JAK/STAT3 signaling following IL-6 ligand–receptor binding has been shown to increase expression of the inhibitors of programmed cell death: Bcl-XL and Mcl-1 [47]. These anti-apoptotic proteins have been shown to attenuate PCD mediated by a

number of cytotoxic agents in MM. In addition to regulation of the Bcl-2 family, STAT3 signaling has been shown to affect p53 expression and expression/suppression of numerous cytokines, chemokines, and growth factors associated with cell survival in a multitude of systems [82–84]. Together, these studies suggest that JAK/STAT3 signaling has a significant influence over MM cell fate. Furthermore, recent work has demonstrated that IL-6 and FN collaborate to selectively enhance JAK/STAT3 conferring a MDR phenotype to co-stimulated MM cells [34]. These results suggest that soluble and physical effectors of the BM microenvironment act in concert conferring MDR and a proliferative advantage to co-stimulated MM cells. Further, a new generation of JAK inhibitors is being developed and has been shown to overcome the protective effects afforded myeloma cells by IL-6, FGF, and BMSCs in response to dex, melphalan, and bortezomib [85, 86].

8.5.4 Src-Family Tyrosine Kinases

The SFKs have been associated with neoplastic transformation since the identification the Rous sarcoma virus. Although first identified in a solid tumor, this family of non-receptor tyrosine kinases has a prominent role in normal hematopoietic proliferation and differentiation. Thirteen SFKs are found exclusively or predominantly in hematopoietic cells [1]. These signaling determinants appear to act as catalysts promoting/enhancing specific signaling cascades. To this end, their role in hematologic malignancies remains an important avenue of investigation. In MM, gp130 and SFKs (Fyn, Lyn, and Hck) were shown to be involved in IL-6 signaling more than demonstrated more than a decade ago [87]. More recently, an SFK (Hck)-binding "acidic peptide" domain has been identified on gp130 necessary for optimal IL-6 signaling in MM cells [62]. Incubation of MM cells with antisense oligonucleotides or pharmacological inhibition of the SFK Lyn attenuated IL-6-mediated proliferation and survival [88]. The SFK Fer has also been directly linked to enhanced STAT3 activity following IL-6 stimulation in other cancer cell models [63]. Together, these data demonstrate a prominent role for SFKs in MM and suggest that this family of tyrosine kinases may provide an appropriate target for novel therapeutic regimens. SFKs may represent an additional signaling determinant elicited by soluble factors of the BM microenvironment. The soluble determinants, however, represent only one component of EM-DR. The physical environment also plays a significant and expanding role in MM therapy resistance.

8.5.5 NF-κB

The last signal transduction cascade to be reviewed involves the nuclear factor (NF)-κB family. The importance of this family of signaling effectors in MM and numerous other hematopoietic malignancies cannot be underscored [89]. The dependence of

intramedullary growth and survival of myeloma cells on classical, non-canonical, and alternative NF-κB signaling pathways has been shown to involve extracellular stimuli (e.g. soluble and physical effectors) as well as heritable genetic alterations in key activating components [89–93]. Within myeloma cells, NF-κB effectors control proliferation, apoptosis, adhesion, migration, and DNA damage repair [27, 50, 53, 94]. Within the context of the BM niche, NF-κB signaling modulates the expression of a number of soluble effectors including IL-6, BAFF, and ARPIL by BMSCs and osteoclasts [32, 38]. Further, this pathway also facilitates increased cellular adhesion to ECM and BMSCs via regulation of ICAM and VCAM [27]. As would be predicted by its role in the expression of determinants listed above, inhibition of NF-κB signaling has been shown to overcome the pro-myeloma aspects of microenvironment produced by BMSCs and in in vivo murine studies [50]. Lastly, the clinical success of the proteasome inhibitor bortezomib is at least partially attributable to the negative regulation of this pathway in the context of BM niche. These results suggest that NF-κB is an important signaling effector(s) within the BM microenvironment and, therefore, a viable target to overcome EM-DR and MRD.

8.6 Direct Cell Contact

The physical components of the *BM* niche have been demonstrated to play a prominent role in MM pathogenesis and MDR. Moreover, data suggest that the physical elements of the microenvironment evolve with progression. Alterations in the expression of the ECM components FN, collagen I, and collagen IV have been shown to correlate with disease state (normal, MGUS vs. frank MM) [95]; thereby providing further evidence that MM and the BM microenvironment are intimately linked. Interactions between cells and their physical environment are mediated by the cell adhesion molecule (CAM) superfamily. CAMs promote communication between the physical environment, cellular architecture, and intracellular signaling cascades. These cell surface molecules include the Ig (immunoglobulin) family, cadherins, selectins, hyaluronate receptors, receptor tyrosine phosphatases, and integrins. These transmembrane receptors coordinate homing, lodging, and differentiation of MM cells in the marrow niche through specific homotypic and heterotypic interactions with environmental ligands [96, 97]. As with soluble factors associated with MM, interactions between MM cells and ECM components or adjacent cells of the BM also confer de novo MDR [47, 98]. Although integrins will be primarily discussed below, numerous other adhesion molecules are also involved in cell adhesion-mediated drug resistance (CAM-DR) including hyaluronan, LFA (lymphocyte-associated function antigen-1), Notch-1/Jagged, Wnt/RhoA, and MMSET gene products [46, 99, 100]. Moreover, these interactions are not limited to cell: ECM interactions. BMSCs, osteoclasts, BM dendritic cells, vascular endothelial cells, and likely immune effectors also support myeloma drug resistance through direct contact [3, 27, 31, 39].

When speaking about myeloma cell adhesion to ECM or adjacent cellular marrow components, it is important to note that at least two levels of response can be separated temporally and by mechanism of activation. Two phases exist, an *early phase*, characterized by rapid dynamic biologic changes, and a *delayed phase*, characterized by secondary signaling and transcriptional events [27, 31]. Within the early phase, posttranslational events result in intracellular redistribution, degradation, and increased stability of critical proteins [34, 36, 101–103]. What is unique about these early events is that they are reversible; within minutes of disassociating from the adherent matrix, myeloma cells return to a drug-sensitive phenotype [36, 101]. The rapid reversal of these events is important when considering adhesion as a target for therapy. As such, only transient alterations in the adhesive phenomenon may be necessary to sensitize myeloma cells to therapy. The delayed events are characterized by transcriptional activity and the production and secretion of soluble effectors such as IL-6, MIP-1α, VEGF, and others (Table 8.1). In turn, these effectors participate in apparent feed-forward paracrine signaling between MM cells and the surrounding cellular milieu facilitating indirect EM-DR.

8.6.1 Integrin-Mediated Adhesion

The role of cell survival and MDR has been best characterized in integrin-mediated adhesion. Integrins are heterodimeric membrane receptors consisting of an α and β subunit ligand-specific binding properties [96]. These cell surface receptors bind extracellular ligands such as FN, vitronectin, laminin, collagens, or other CAMs. Integrins do not have intrinsic kinase activity and, therefore, are dependent upon associated factors such as focal adhesion kinase, RAFTK, PI3-kinase, ILK (integrin-linked kinase), PINCH (particularly interesting new cysteine–histidine-rich protein), Nck2 (non-catalytic *region of* tyrosine kinase adaptor protein 2) to elicit intracellular signaling [97]. The organization of intracellular protein complex facilitates the activation of signaling cascades and cytoskeletal changes following the integrin cross-linking that modulates cell growth, differentiation, migration, and survival (Fig. 8.3b). Integrin-specific CAM-DR in MM was initially described in studies demonstrating that β1-integrin-specific adhesion of the MM cell lines to FN conferred resistance to several classes of chemotherapeutic agents both in cell lines and in patient samples [47, 104–106]. Evidence of CAM-DR was noted in studies demonstrating elevated expression of VLA-4 (α4β1 integrin receptors), ICAM, and VCAM-1 in MM patient samples exposed to chemotherapy relative to treatment-naive patients [98]. More recently, β7 integrins have been demonstrated to similarly promote myeloma growth, survival, migration, and drug resistance [107]. From these studies, it is apparent that targeting determinants of CAM-DR may lead to the development of clinical regimens to circumventing de novo MDR and MRD. Below we will review a number of cellular determinants of CAM-DR.

8.6.1.1 Bim proteolytic processing

β1-integrin-mediated adhesion to immobilized FN confers a survival advantage to adherent myeloma cell lines in response to traditional therapeutic agents. Integrin binding has a number of documented mechanisms facilitating resistance. One CAM-DR determinant is the pro-apoptotic Bcl-2 family member Bim [108]. The Bcl-2 family of proteins plays a crucial role in apoptosis via modulation of mitochondrial integrity. Studies have outlined the complex sequence of interactions by which Bcl-2 family proteins modulate ted apoptosis. This family consists of three major subgroups: anti-apoptotic members [Bcl-2, Bcl-$X_{L,}$ Bcl-w, myeloid cell leukemia [109]-1, and Bcl-2-related protein (Bfl/A1)] that contain four Bcl-2 homology domains (BHDs); pro-apoptotic mitochondrial-membrane-associated members [Bcl-2-related ovarian killer [110], Bcl-2-antagonist/killer (Bak), and Bcl-2-associated X protein (Bax)] that contain three BHDs; and pro-apoptotic ligands that contain only a single BHD [Bcl-2-like 11, apoptosis facilitator (Bim), Bcl-2-interacting killer, apoptosis inducing [79], Bcl-2-associated agonist of cell death (Bad), Bh3 interacting domain death agonist (Bid), and phorbol-12-myristate-13-acetate-induced protein 1 (Noxa)]. The interplay between these factors controls the fate of the cell. Following cytotoxic insult, Bim targets the mitochondria facilitating mitochondrial-dependent apoptosis. However, in FN-adherent cells, Bim is downregulated via a dynamic proteasome-dependent process. To this end, posttranslational regulation of the pro-apoptotic effector Bim represents a novel mechanism of CAM-DR [108].

8.6.1.2 Intracellular Redistribution of Topoisomerase IIα and IIβ

CAM-DR has also been shown to reduce drug cytotoxicity via alterations in drug target. Topoisomerase IIβ is involved in repair of DNA damage induced by DNA specific toxins including topoisomerase poisons and the alkylating agent melphalan [111]. FN-mediated adhesion protects adhered leukemic cells from mitoxantrone- and etoposide-induced DNA double-strand breaks [106]. This integrin-mediated reduction in DNA damage paralleled a decrease in topoisomerase IIβ enzymatic activity, salt extractability, and altered nuclear localization. These data demonstrate a mechanism by which environmental effectors may regulate sensitivity to cytotoxic agents via the subcellular redistribution of a key chemotherapeutic target, topoisomerase IIβ. Topoisomerase IIα intracellular localization is also controlled by the microenvironmental factors. In the setting of increased myeloma cell density, primed for cell-to-cell contact, myeloma cells are resistant to chemotherapeutics [103, 112]. This resistance was causally associated with the Crm1-dependent nuclear export of topoisomerase IIα [103].

8.6.1.3 Increased $p27^{kip1}$

β1-integrin-mediated adhesion to immobilized FN also confers a survival advantage to adherent MM cell lines to cytotoxic agents via a $p27^{kip1}$-dependent resistance

to etoposide and cell cycle arrest [113]. The decrease in apoptosis and growth arrest following FN adhesion occurred through the posttranslational regulation of $p27^{kip1}$ protein expression. In accordance with these studies, lymphoma cell adhesion via integrins was demonstrated to regulate levels of $p27^{kip1}$ via modulation of the SCF/Skp2 ubiquitin ligase pathway [114]. In the lymphoma model, cell adhesion was shown to posttranslationally increase levels of cdh1, an activating component of the anaphase-promoting complex. This ubiquitin ligase can target Skp2 for ubiquitinization and degradation. Skp2 degradation leads to stabilization and maintenance of $p27^{kip1}$ protein levels and cell cycle arrest [14]. Together, these studies suggest that cell adhesion may facilitate $p27^{kip1}$-mediated drug resistance via a proteasome ubiquitin pathway.

8.6.1.4 Intracellular Redistribution of $cFLIP_L$

Adhesion to FN also affects physiologic determinants of apoptosis. CD95/Fas and TRAILR2/DR5 are two members of the TNF superfamily that facilitate apoptosis. MM cell adhesion to FN controls the intracellular localization of the anti-apoptotic protein c-FLIP [115]. Only in adherent cells is c-FLIP able to associate with FADD and CD95 to block the apoptotic signal. In contrast, when cells are maintained in suspension, c-FLIP is sequestered unable to block death receptor-mediated signaling. These data, together with evidence of alterations on protein stability (topoisomerase IIα, β, Bim, and $p27^{kip1}$), suggest that adhesion-orchestrated posttranslational regulation is an important mechanism by which the physical environment confers resistance to cytotoxic stress and MDR.

8.6.2 Notch1/Jagged

In addition to integrins, reports have identified Notch-1 and Jagged signaling as an important modulator of EM-DR via BMSCs to adherent MM cells. MM and BMSC interactions are complex making the identification of specific determinants of EM-DR arduous. However, Notch-1, a large heterodimeric transmembrane receptor, and its interactions with ligand Jagged have been demonstrated to confer an MDR phenotype to BM stromal interacting MM cells [116]. Notch-1-specific signaling protects MM and other malignant hematopoietic cells from melphalan and mitoxantrone via an increase in $p21^{Cip1/WAF1}$ protein levels. Regulation of $p21^{Cip1/WAF1}$ expression has been proposed to occur similarly to that of $p27^{kip1}$ as discussed above. More recently, attenuation of Notch-1 signaling via gamma-secretase inhibitors was shown to (1) facilitate apoptosis in MM cell lines and (2) enhance melphalan- and doxorubicin-mediated cell death in in vitro and in vivo (xenograft and SCID-hu) models [100]. Together, these experiments suggest that Notch-1 signaling is an important target of EM-DR with potential therapeutic implications.

8.7 A Complex Network of Survival Signals

Soluble and physical determinants of the BM microenvironment independently confer the EM-DR phenotype. However, logic dictates that these soluble and physical environmental effectors function in concert. This network involves autocrine/paracrine signaling as well as regulatory events mediated by adhesion of myeloma cells to BMSCs, osteoclasts, osteoblasts, MSCs, immune effectors, and endothelial cells (Fig. 8.2) [27, 117]. These cellular interactions are important catalysts for the production of the numerous soluble factors [3, 118, 119]. However, it is apparent that the maximal effect of EM-DR is not mediated by soluble or physical effectors alone. Instead, these factors cooperate in conferring EM-DR. Evidence for this has been provided in studies examining the anti-apoptotic nature of specific co-culturing conditions between myeloma cell and BMSCs [116, 120]. In these studies, the

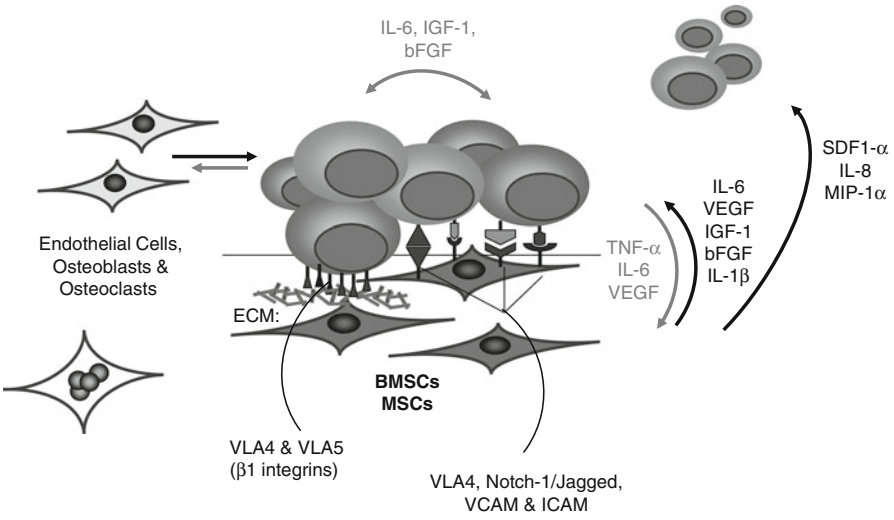


Fig. 8.2 *The bone marrow niche is composed of a network of extracellular determinants*. The BM microenvironment plays a key role in the disease pathology of MM. It is rich in cytokines, chemokines, growth factors, and adhesive matrices that facilitate MM cell homing, proliferation, and survival. The protective determinants of the BM consist of both soluble factors and physical factors. These factors include cytokines: IL-1β, IL-3, IL-15, IL-21, TNF-α, OSM, LIF; chemokines: IL-8, CXCL12, MIP1-α; growth factors: VEGF (vascular endothelial growth factor), FGF (family), IGF-1 (insulin-like GF-1), HGF (hepatocyte GF); and other pro-myeloma factors: Dkk-1 (Dickkopf-1), RANKL [receptor activator of nuclear factor kappaB (NF-κB) ligand], BAFF (B cell activation growth factor), Wnt (family), and TGF-β (transforming GF) among others [27, 32, 33]. In addition to direct proliferative and survival effects, soluble factors also impart the adhesion of myeloma cells to adjacent cellular components (stromal cells, dendritic cells, MSCs, macrophages, and osteoclasts) and extracellular matrices (fibronectin, collagens, vitronectin, GAG, and laminins) via a host of adhesion molecules. The most prominent being VLA-4 (α4/β1/CD49d/CD29), VLA-5 (α5/β1/CD49e/CD29), CD44, VCAM-1, ICAM (CD54), NCAM (CD56), CD74, and CS-1 (CD2-subset-1) among others [3, 27, 31]. Extracellular stimulation by soluble factors or direct adhesion facilitates networks of intracellular cascades

degree of chemotherapy resistance was dependent upon the co-culture condition examined. Myeloma cells co-cultured in contact with BMSCs were protected to greater degree than cells co-cultured without contact. These results demonstrate the existence of at least two environmental networks elicited by interactions between MM cells and BMSCs: a network involving anti-apoptotic paracrine signaling and a network involving the conjunction of soluble and physical effectors, with the combination providing a more pronounced survival advantage [27, 49].

An explanation of these findings may be suggested by studies examining the intracellular consequences of co-stimulation [34]. These data demonstrate that signaling events are specifically enhanced relative to those elicited by a single effector. A report examining the intracellular signaling following stimulation with IL-6 alone, FN adhesion alone, or their combination demonstrated the complexity of converging extracellular determinants [34]. The collaboration between IL-6 and FN adhesion resulted in a selective enhancement of JAK/STAT3 phosphorylation and activity, but not Akt or ERK1/2 phosphorylation. Co-stimulated cells were afforded both a proliferative and an MDR advantage. Further, this collaboration paralleled increased gp130 complex phosphorylation via a novel IL-6-independent pre-association of STAT3 (unphosphorylated) with gp130 when cells are adhered to FN. Together, these results suggest that examination of crosstalk between intracellular signaling networks may identify specific components, which contribute to a greater (or lesser) degree to therapy resistance and proliferation (i.e., more in vivo-like conditions, where cells are not modulated by a single determinant). To this end, identification of prominent signaling molecules under co-stimulatory conditions may direct us toward more appropriate drug targets.

8.7.1 Therapeutic Implications

As discussed above, EM-DR and MRD are prominent hurdles in the treatment failure. To this end, successful treatment may involve correctly identifying modalities that target the myeloma microenvironment and/or attendant signaling cascades. The novel agents bortezomib, thalidomide, and lenalidomide have had a significant impact on the treatment of this disease [2]. These therapeutic compounds have been demonstrated to function, at least in part, via a modulation of the microenvironment. Bortezomib is a direct inhibitor of the 26S proteasome and has a number of *indirect* effects on the microenvironment through the downregulation of key paracrine effectors. Proteasome inhibition has been shown to overcome EM-DR in preclinical studies with cell lines and patient samples [2]. Bortezomib regulates the expression of numerous cytokines and growth factors. Bortezomib has been shown to inhibit angiogenesis via a modulation of VEGF and bFGF, as well as inhibit cellular adhesion via deregulation of VCAM and ICAM [2]. Gene expression profiling of myeloma cells treated with bortezomib demonstrated decreased production of α4 integrin/CD49d consistent with a role in targeting EM-DR [121]. Thalidomide and the immune modulatory compound lenalidomide also have anti-EM-DR properties.

The proposed action of thalidomide primarily involves the downregulation of VEGF and associated angiogenic factors [2]. Recent phase II studies using the VEGFR inhibitor pazopanib suggests that VEGF antagonism may not be sufficient as a single-agent therapy [122]. Additionally, lenalidomide also has significant clinical benefit when coupled with dexamethasone [2], likely secondary to its proposed activity of fortifying antitumor immune system. However, as with bortezomib and thalidomide, lenalidomide also demonstrates multi-targeted effects on the BM microenvironment (cytokine and growth factor expression, inhibition of angiogenesis, and inhibition of cellular adhesion). Unfortunately, even with the widespread utilization of these therapeutics, myeloma remains incurable. As such, continued investigation into therapies to compliment their activity is required.

With our greater appreciation for the role of EM-DR, a large number of agents have been designed to targeting determinants of the BM niche. Small-molecule inhibitors of IL-6 and IL-6-dependent signaling have been identified. Early studies with Sant7 demonstrated positive preclinical results [123, 124]. More recently, monoclonal antibody therapy with the chimeric monoclonal antibody (CNTO 328) specific to IL-6 has shown preclinical success in myeloma cell lines and patient samples. A phase II study in previously treated patients demonstrated a 57% overall response rate following therapy with the combination of CNTO 328 and bortezomib, suggesting that this anti-IL-6 chimeric immunoglobulin may have anti-myeloma activity [125].

Drugs targeting other extracellular determinants are also being evaluated. Initially examined in the setting of HIV and then stem cell harvesting [126], the CXCR4 inhibitor AMD3100/Plerixafor has been shown to attenuate myeloma–BMSC communication. These effects translated to enhance cytotoxicity of bortezomib [17, 24]. AMD3100 acts to interfere with homing of cells to the BM, but more importantly its effects may be to facilitate a deadhesion phenomenon, thereby attenuating the resistance conferred to resident myeloma cells by the physical microenvironment. AMD3100 has also been examined in other hematologic and non-hematologic malignancies [127, 128]. A humanized monoclonal antibody to CXCR4 is also being evaluated in clinical and preclinical studies (BMS983564). The FGFR- and VEGFR-*specific* receptor tyrosine kinase inhibitor (BIBF100) was examined in MM cell lines and patient samples [129]. The cytotoxicity of bortezomib and dexamethasone was enhanced when combined with BIBF100 in poor-risk patient samples (and cell lines) carrying t(4:14) (FGFR3/MMSET; IgH translocations) and t(14:16) (c-maf; IgH translocations—associated with c-maf-mediated VEGF signaling) [129]. Sorafenib, a putative VEGFR tyrosine kinase inhibitor, has shown preclinical promise in myeloma [130].

The physical microenvironment is also being targeted with novel therapeutics (Fig. 8.3b). The design of compounds to target β1 integrins or other mediators of

Fig. 8.3 (continued) the activation of signaling cascades including SFK, PI3-kinase, Ras/Raf–ERK1/2 kinase, and NF-κB. These cascades have been directly and indirectly linked to cytoskeletal changes following the integrin cross-linking and modulation of cell growth, differentiation, migration, and survival. To this end, inhibitors of these cellular processes (extracellular or intracellular) may have anti-myeloma activity

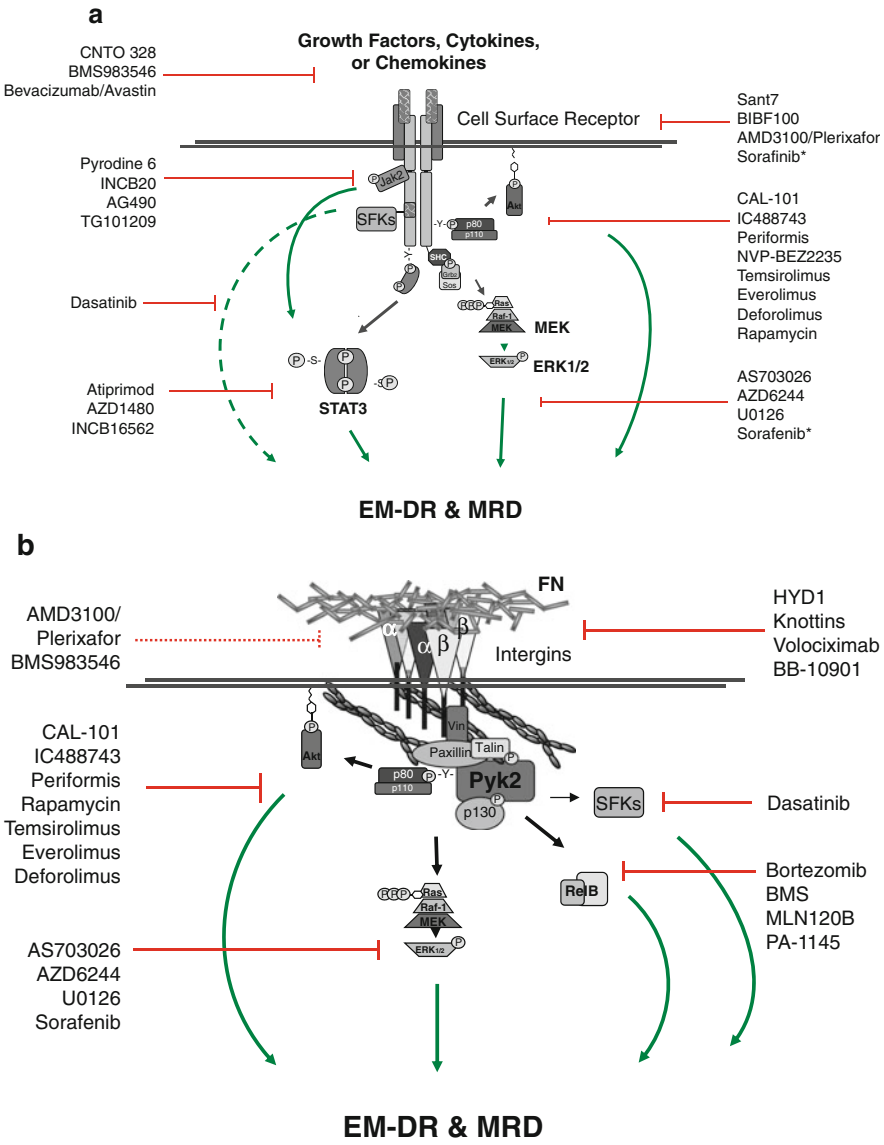


Fig. 8.3 *Extracellular and Intracellular Signaling Pathways are Targets for Novel Therapeutics*. Numerous extracellular determinants of the BM initiate intracellular signaling cascades culminating in EM-DR and MRD. (**a**) This figure depicts a general outline for signaling modulated by cell membrane receptors (cytokines, GFs, and chemokines). A new generation of potential therapies is being examined. (**b**) The role of cell survival and MDR has been best characterized in integrin-mediated adhesion. Integrins are a family of single-pass heterodimeric membrane receptors. Integrin receptors lack intrinsic kinase activity and so depend on associated factors such as focal adhesion kinase, RAFTK, PI3-kinase, ILK (integrin-linked kinase), PINCH (particularly interesting new cysteine-histidine rich protein), Nck2 (non-catalytic *region of* tyrosine kinase adaptor protein 2) to elicit intracellular signaling. The organization of intracellular protein complex facilitates

adhesion between tumor cells and the physical environment may provide therapeutic benefit. In AML, VLA-4 (α4β1)-specific antibodies were shown to potentiate the antitumor effects of cytarabine in vivo by reducing MRD [131]. Volociximab, a chimeric monoclonal antibody to VLA-5 (α5β1), was tested in solid tumors demonstrating mild clinical benefit and was well tolerated [132]. These early reports indicate that integrin-direct antibody therapy may be an important adjunct in targeting MRD. Integrin–ECM interactions involve the recognition of a specific three amino acid sequence, RGD (arginine, glycine, and aspartate). Linear peptides, cyclic peptides, and peptidomimetics have been designed to mimic this peptide sequence to disrupt integrin-mediated adhesion [133]. Recently, a new group of RGD-blocking compounds has been identified. The cystine knot peptides or *knottins* have been engineered demonstrating nanomolar affinity for multiple integrin heterodimers [133]. Another example is the decapeptide HYD1, synthesized based on the RGD amino acid motif, which has been shown to attenuate VLA-4 integrin-mediated adhesion to FN in multiple cancer cell models. Interestingly, incubation of myeloma cell lines and patient samples with HYD1 not only abrogated adhesion to ECM but also induced myeloma cell necrosis (caspase-independent death) [134]. These results demonstrate that inhibition of integrin-mediated adhesion may have direct (or indirect) anti-myeloma effects and reveal the potential of therapies designed to target the physical microenvironment in cancer therapy.

In the complex milieu of the BM, cell-signaling cascades are also potential therapeutic targets (Fig. 8.3a, b). Pyridone 6, a reversible ATPase inhibitor, blocks JAK activity arresting growth of MM cells and patient samples with constitutive JAK/STAT3 activity [135]. Additional JAK inhibitors including INCB20, AZD1480, and INCB16562 have been shown to attenuate MM cell growth in response to IL-6 and BMSCs [69] PI3-kinase, Akt, and mTOR are also promising targets for therapeutic intervention [27, 73, 74, 78]. Ras/Raf–MEK–ERK1/2 signaling, especially MEK, is another pathway with a growing number of promising inhibitory compounds with anti-EM-DR properties [64, 65, 67, 136]. Lastly, SFK inhibitors are also being examined in MM. Preclinical data with the tyrosine kinase inhibitor dasatinib demonstrated growth arrest and synergism with conventional and novel therapeutics in MM cell lines [137]. By no means is this a complete listing of novel anti-EM-DR compounds, but it demonstrates the principles behind drug design in the context of the microenvironment.

The inhibitors discussed above target both extracellular and intracellular determinants of the BM niche. The hope is that we can identify the appropriate factors within the complex network of the tumor cell microenvironment to target. To this end, we may divine therapies to overcome the coordinated effort between MM cells and the microenvironment. In so doing, we may be able interrupt the sequence of events (de novo and acquired) facilitating MRD culminating in therapy resistance. However, it is important to note that with the significant heterogeneity of signaling factors and transduction pathways within the BM niche, we will need to design combination therapies with targeted agents. To this end, targeting of multiple pathways either simultaneously or in sequence may be the only measure by which to overcome the sanctuary of the BM milieu.

In conclusion, the treatment of myeloma remains in the midst of a revolution. For the first time in decades, we have novel therapeutic modalities at our disposal with significant clinical benefit (thalidomide, lenalidomide, and bortezomib) due to the multi-targeted activities. As discussed, the refractory nature of this malignancy stems from the protective nature of BM microenvironment (at least in part). Resident MM cells are afforded sanctuary from therapeutic insult resulting in MRD. To this end, improved PFS and OS will require the development of microenvironment-targeted agents and the rational combination of these drugs to combat EM-DR and overcome MRD.

References

1. Gertz MA (2008) New targets and treatments in multiple myeloma: Src family kinases as central regulators of disease progression. Leuk Lymphoma 49(12):2240–2245
2. Richardson PG, Hideshima T, Mitsiades C, Anderson KC (2007) The emerging role of novel therapies for the treatment of relapsed myeloma. J Natl Compr Canc Netw 5(2):149–162
3. Mitsiades CS, McMillin DW, Klippel S, Hideshima T, Chauhan D, Richardson PG et al (2007) The role of the bone marrow microenvironment in the pathophysiology of myeloma and its significance in the development of more effective therapies. Hematol Oncol Clin North Am 21(6):1007–1034, vii–viii
4. Durie BG, Harousseau JL, Miguel JS, Blade J, Barlogie B, Anderson K et al (2006) International uniform response criteria for multiple myeloma. Leukemia 20(9):1467–1473
5. Cavo M, Terragna C, Martinelli G, Ronconi S, Zamagni E, Tosi P et al (2000) Molecular monitoring of minimal residual disease in patients in long-term complete remission after allogeneic stem cell transplantation for multiple myeloma. Blood 96(1):355–357
6. Martinelli G, Terragna C, Zamagni E, Ronconi S, Tosi P, Lemoli R et al (2000) Polymerase chain reaction-based detection of minimal residual disease in multiple myeloma patients receiving allogeneic stem cell transplantation. Haematologica 85(9):930–934
7. Fenk R, Ak M, Kobbe G, Steidl U, Arnold C, Korthals M et al (2004) Levels of minimal residual disease detected by quantitative molecular monitoring herald relapse in patients with multiple myeloma. Haematologica 89(5):557–566
8. Bakkus MH, Bouko Y, Samson D, Apperley JF, Thielemans K, Van Camp B et al (2004) Post-transplantation tumour load in bone marrow, as assessed by quantitative ASO-PCR, is a prognostic parameter in multiple myeloma. Br J Haematol 126(5):665–674
9. Attal M, Harousseau JL, Facon T, Guilhot F, Doyen C, Fuzibet JG et al (2003) Single versus double autologous stem-cell transplantation for multiple myeloma. N Engl J Med 349(26): 2495–2502
10. Barlogie B, Anaissie E, van Rhee F, Haessler J, Hollmig K, Pineda-Roman M et al (2007) Incorporating bortezomib into upfront treatment for multiple myeloma: early results of total therapy 3. Br J Haematol 138(2):176–185
11. San-Miguel JF, Mateos MV (2009) How to treat a newly diagnosed young patient with multiple myeloma. Hematology Am Soc Hematol Educ Program 2009:555–565
12. Nair B, van Rhee F, Shaughnessy JD Jr, Anaissie E, Szymonifka J, Hoering A et al (2010) Superior results of Total Therapy 3 (2003-33) in gene expression profiling-defined low-risk multiple myeloma confirmed in subsequent trial 2006-66 with VRD maintenance. Blood 115(21):4168–4173
13. Kumar A, Kharfan-Dabaja MA, Glasmacher A, Djulbegovic B (2009) Tandem versus single autologous hematopoietic cell transplantation for the treatment of multiple myeloma: a systematic review and meta-analysis. J Natl Cancer Inst 101(2):100–106

14. van de Velde HJ, Liu X, Chen G, Cakana A, Deraedt W, Bayssas M (2007) Complete response correlates with long-term survival and progression-free survival in high-dose therapy in multiple myeloma. Haematologica 92(10):1399–1406
15. Paiva B, Martinez-Lopez J, Vidriales MB, Mateos MV, Montalban MA, Fernandez-Redondo E et al (2011) Comparison of immunofixation, serum free light chain, and immunophenotyping for response evaluation and prognostication in multiple myeloma. J Clin Oncol 29(12):1627–1633
16. Shain KH, Landowski TH, Dalton WS (2000) The tumor microenvironment as a determinant of cancer cell survival: a possible mechanism for de novo drug resistance. Curr Opin Oncol 12(6):557–563
17. Alsayed Y, Ngo H, Runnels J, Leleu X, Singha UK, Pitsillides CM et al (2007) Mechanisms of regulation of CXCR4/SDF-1 (CXCL12)-dependent migration and homing in multiple myeloma. Blood 109(7):2708–2717
18. van de Donk NW, Lokhorst HM, Bloem AC (2005) Growth factors and antiapoptotic signaling pathways in multiple myeloma. Leukemia 19(12):2177–2185
19. Shapiro-Shelef M, Calame K (2005) Regulation of plasma-cell development. Nat Rev Immunol 5(3):230–242
20. Shapiro-Shelef M, Calame K (2004) Plasma cell differentiation and multiple myeloma. Curr Opin Immunol 16(2):226–234
21. Klein U, Dalla-Favera R (2008) Germinal centres: role in B-cell physiology and malignancy. Nat Rev Immunol 8(1):22–33
22. Shaffer AL, Shapiro-Shelef M, Iwakoshi NN, Lee AH, Qian SB, Zhao H et al (2004) XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. Immunity 21(1):81–93
23. Sciammas R, Davis MM (2004) Modular nature of Blimp-1 in the regulation of gene expression during B cell maturation. J Immunol 172(9):5427–5440
24. Azab AK, Azab F, Blotta S, Pitsillides CM, Thompson B, Runnels JM et al (2009) Rho-A and Rac-1 GTPases play major and differential roles in SDF1-induced cell adhesion and chemotaxis in multiple myeloma. Blood 114(3):619–629
25. Azab AK, Runnels JM, Pitsillides C, Moreau AS, Azab F, Leleu X et al (2009) CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy. Blood 113(18):4341–4351
26. Kyle RA, Rajkumar SV (2008) Multiple myeloma. Blood 111(6):2962–2972
27. Podar K, Chauhan D, Anderson KC (2009) Bone marrow microenvironment and the identification of new targets for myeloma therapy. Leukemia 23(1):10–24
28. Kline M, Donovan K, Wellik L, Lust C, Jin W, Moon-Tasson L et al (2007) Cytokine and chemokine profiles in multiple myeloma; significance of stromal interaction and correlation of IL-8 production with disease progression. Leuk Res 31(5):591–598
29. Pilarski LM, Pilarski PM, Belch AR (2010) Multiple myeloma may include microvessel endothelial cells of malignant origin. Leuk Lymphoma 51(4):592–597
30. Edwards CM, Zhuang J, Mundy GR (2008) The pathogenesis of the bone disease of multiple myeloma. Bone 42(6):1007–1013
31. Meads MB, Gatenby RA, Dalton WS (2009) Environment-mediated drug resistance: a major contributor to minimal residual disease. Nat Rev 9(9):665–674
32. Gunn WG, Conley A, Deininger L, Olson SD, Prockop DJ, Gregory CA (2006) A crosstalk between myeloma cells and marrow stromal cells stimulates production of DKK1 and interleukin-6: a potential role in the development of lytic bone disease and tumor progression in multiple myeloma. Stem Cells 24(4):986–991
33. Mahtouk K, Moreaux J, Hose D, Reme T, Meissner T, Jourdan M et al (2010) Growth factors in multiple myeloma: a comprehensive analysis of their expression in tumor cells and bone marrow environment using Affymetrix microarrays. BMC Cancer 10:198
34. Shain KH, Yarde DN, Meads MB, Huang M, Jove R, Hazlehurst LA et al (2009) Beta1 integrin adhesion enhances IL-6-mediated STAT3 signaling in myeloma cells: implications for microenvironment influence on tumor survival and proliferation. Cancer Res 69(3):1009–1015

35. Ishikawa H, Tsuyama N, Obata M, Kawano M (2006) Mitogenic signals initiated via interleukin-6 receptor complexes in cooperation with other transmembrane molecules in myelomas. J Clin Exp Hematop 46(2):55–66
36. Shain KH, Dalton WS (2001) Cell adhesion is a key determinant in de novo multidrug resistance (MDR): new targets for the prevention of acquired MDR. Mol Cancer Ther 1(1):69–78
37. McMillin DW, Delmore J, Weisberg E, Negri JM, Geer DC, Klippel S et al (2010) Tumor cell-specific bioluminescence platform to identify stroma-induced changes to anticancer drug activity. Nat Med 16(4):483–489
38. Yaccoby S (2010) Advances in the understanding of myeloma bone disease and tumour growth. Br J Haematol 149(3):311–321
39. Roodman GD (2010) Targeting the bone microenvironment in multiple myeloma. J Bone Miner Metab 28(3):244–250
40. Bartel TB, Haessler J, Brown TL, Shaughnessy JD Jr, van Rhee F, Anaissie E et al (2009) F18-fluorodeoxyglucose positron emission tomography in the context of other imaging techniques and prognostic factors in multiple myeloma. Blood 114(10):2068–2076
41. Walker R, Barlogie B, Haessler J, Tricot G, Anaissie E, Shaughnessy JD Jr et al (2007) Magnetic resonance imaging in multiple myeloma: diagnostic and clinical implications. J Clin Oncol 25(9):1121–1128
42. Rajkumar SV, Greipp PR (2001) Angiogenesis in multiple myeloma. Br J Haematol 113(3):565
43. Du W, Hattori Y, Hashiguchi A, Kondoh K, Hozumi N, Ikeda Y et al (2004) Tumor angiogenesis in the bone marrow of multiple myeloma patients and its alteration by thalidomide treatment. Pathol Int 54(5):285–294
44. Markovic O, Marisavljevic D, Cemerikic V, Vidovic A, Perunicic M, Todorovic M et al (2008) Expression of VEGF and microvessel density in patients with multiple myeloma: clinical and prognostic significance. Med Oncol 25(4):451–457
45. Qiang YW, Barlogie B, Rudikoff S, Shaughnessy JD Jr (2008) Dkk1-induced inhibition of Wnt signaling in osteoblast differentiation is an underlying mechanism of bone loss in multiple myeloma. Bone 42(4):669–680
46. Lauring J, Abukhdeir AM, Konishi H, Garay JP, Gustin JP, Wang Q et al (2008) The multiple myeloma associated MMSET gene contributes to cellular adhesion, clonogenic growth, and tumorigenicity. Blood 111(2):856–864
47. Li ZW, Dalton WS (2006) Tumor microenvironment and drug resistance in hematologic malignancies. Blood Rev 20(6):333–342
48. Pollett JB, Trudel S, Stern D, Li ZH, Stewart AK (2002) Overexpression of the myeloma-associated oncogene fibroblast growth factor receptor 3 confers dexamethasone resistance. Blood 100(10):3819–3821
49. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC (2007) Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. Nat Rev 7(8):585–598
50. Yasui H, Hideshima T, Richardson PG, Anderson KC (2006) Novel therapeutic strategies targeting growth factor signalling cascades in multiple myeloma. Br J Haematol 132(4):385–397
51. Burger JA, Kipps TJ (2006) CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. Blood 107(5):1761–1767
52. Burns JM, Summers BC, Wang Y, Melikian A, Berahovich R, Miao Z et al (2006) A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. J Exp Med 203(9):2201–2213
53. Teicher BA, Fricker SP (2010) CXCL12 (SDF-1)/CXCR4 pathway in cancer. Clin Cancer Res 16(11):2927–2931
54. Sipkins DA, Wei X, Wu JW, Runnels JM, Cote D, Means TK et al (2005) In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature 435(7044):969–973
55. Sanz-Rodriguez F, Hidalgo A, Teixido J (2001) Chemokine stromal cell-derived factor-1alpha modulates VLA-4 integrin-mediated multiple myeloma cell adhesion to CS-1/fibronectin and VCAM-1. Blood 97(2):346–351

56. Hartmann TN, Burger JA, Glodek A, Fujii N, Burger M (2005) CXCR4 chemokine receptor and integrin signaling co-operate in mediating adhesion and chemoresistance in small cell lung cancer (SCLC) cells. Oncogene 24(27):4462–4471
57. Lu DY, Tang CH, Yeh WL, Wong KL, Lin CP, Chen YH et al (2009) SDF-1alpha up-regulates interleukin-6 through CXCR4, PI3K/Akt, ERK, and NF-kappaB-dependent pathway in microglia. Eur J Pharmacol 613(1–3):146–154
58. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F (2003) Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J 374(Pt 1):1–20
59. Li WX (2008) Canonical and non-canonical JAK-STAT signaling. Trends Cell Biol 18(11):545–551
60. Raza A, Reeves JA, Feldman EJ, Dewald GW, Bennett JM, Deeg HJ et al (2008) Phase 2 study of lenalidomide in transfusion-dependent, low-risk, and intermediate-1 risk myelodysplastic syndromes with karyotypes other than deletion 5q. Blood 111(1):86–93
61. Feldman EJ, Cortes J, DeAngelo DJ, Holyoake T, Simonsson B, O'Brien SG et al (2008) On the use of lonafarnib in myelodysplastic syndrome and chronic myelomonocytic leukemia. Leukemia 22(9):1707–1711
62. Hausherr A, Tavares R, Schaffer M, Obermeier A, Miksch C, Mitina O et al (2007) Inhibition of IL-6-dependent growth of myeloma cells by an acidic peptide repressing the gp130-mediated activation of Src family kinases. Oncogene 26(34):4987–4998
63. Zoubeidi A, Rocha J, Zouanat FZ, Hamel L, Scarlata E, Aprikian AG et al (2009) The Fer tyrosine kinase cooperates with interleukin-6 to activate signal transducer and activator of transcription 3 and promote human prostate cancer cell growth. Mol Cancer Res 7(1):142–155
64. Tai YT, Fulciniti M, Hideshima T, Song W, Leiba M, Li XF et al (2007) Targeting MEK induces myeloma-cell cytotoxicity and inhibits osteoclastogenesis. Blood 110(5): 1656–1663
65. Kim K, Kong SY, Fulciniti M, Li X, Song W, Nahar S et al (2010) Blockade of the MEK/ERK signalling cascade by AS703026, a novel selective MEK1/2 inhibitor, induces pleiotropic anti-myeloma activity in vitro and in vivo. Br J Haematol 149(4):537–549
66. Pei XY, Dai Y, Tenorio S, Lu J, Harada H, Dent P et al (2007) MEK1/2 inhibitors potentiate UCN-01 lethality in human multiple myeloma cells through a Bim-dependent mechanism. Blood 110(6):2092–2101
67. Annunziata CM, Hernandez L, Davis RE, Zingone A, Lamy L, Lam LT et al (2011) A mechanistic rationale for MEK inhibitor therapy in myeloma based on blockade of MAF oncogene expression. Blood 117(8):2396–2404
68. Popovic R, Licht JD (2011) MEK and MAF in myeloma therapy. Blood 117(8):2300–2302
69. Burger R, Le Gouill S, Tai YT, Shringarpure R, Tassone P, Neri P et al (2009) Janus kinase inhibitor INCB20 has antiproliferative and apoptotic effects on human myeloma cells in vitro and in vivo. Mol Cancer Ther 8(1):26–35
70. Harvey RD, Lonial S (2007) PI3 kinase/AKT pathway as a therapeutic target in multiple myeloma. Future Oncol 3(6):639–647
71. Younes H, Leleu X, Hatjiharissi E, Moreau AS, Hideshima T, Richardson P et al (2007) Targeting the phosphatidylinositol 3-kinase pathway in multiple myeloma. Clin Cancer Res 13(13):3771–3775
72. Hideshima T, Catley L, Raje N, Chauhan D, Podar K, Mitsiades C et al (2007) Inhibition of Akt induces significant downregulation of survivin and cytotoxicity in human multiple myeloma cells. Br J Haematol 138(6):783–791
73. Ikeda H, Hideshima T, Fulciniti M, Perrone G, Miura N, Yasui H et al (2010) PI3K/p110{delta} is a novel therapeutic target in multiple myeloma. Blood 116(9):1460–1468
74. Lannutti BJ, Meadows SA, Herman SE, Kashishian A, Steiner B, Johnson AJ et al (2011) CAL-101, a p110delta selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signaling and cellular viability. Blood 117(2):591–594
75. Chowdhury S, Choueiri TK (2009) Recent advances in the systemic treatment of metastatic papillary renal cancer. Expert Rev Anticancer Ther 9(3):373–379

76. Figlin RA, Brown E, Armstrong AJ, Akerley W, Benson AB III, Burstein HJ et al (2008) NCCN Task Force Report: mTOR inhibition in solid tumors. J Natl Compr Canc Netw 6(Suppl 5):S1–S20, quiz S1–S2
77. Bhaskar PT, Hay N (2007) The two TORCs and Akt. Dev Cell 12(4):487–502
78. Hoang B, Frost P, Shi Y, Belanger E, Benavides A, Pezeshkpour G et al (2010) Targeting TORC2 in multiple myeloma with a new mTOR kinase inhibitor. Blood 116(22):4560–4568
79. McMillin DW, Ooi M, Delmore J, Negri J, Hayden P, Mitsiades N et al (2009) Antimyeloma activity of the orally bioavailable dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235. Cancer Res 69(14):5835–5842
80. Ghobrial IM, Weller E, Vij R, Munshi NC, Banwait R, Bagshaw M et al (2011) Weekly bortezomib in combination with temsirolimus in relapsed or relapsed and refractory multiple myeloma: a multicentre, phase 1/2, open-label, dose-escalation study. Lancet Oncol 12(3):263–272
81. Campbell PJ, Green AR (2006) The myeloproliferative disorders. N Engl J Med 355(23):2452–2466
82. Niu G, Wright KL, Ma Y, Wright GM, Huang M, Irby R et al (2005) Role of Stat3 in regulating p53 expression and function. Mol Cell Biol 25(17):7432–7440
83. Wang T, Niu G, Kortylewski M, Burdelya L, Shain K, Zhang S et al (2004) Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. Nat Med 10(1):48–54
84. Yu H, Kortylewski M, Pardoll D (2007) Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nat Rev Immunol 7(1):41–51
85. Scuto A, Krejci P, Popplewell L, Wu J, Wang Y, Kujawski M et al (2011) The novel JAK inhibitor AZD1480 blocks STAT3 and FGFR3 signaling, resulting in suppression of human myeloma cell growth and survival. Leukemia 25(3):538–550
86. Li J, Favata M, Kelley JA, Caulder E, Thomas B, Wen X et al (2010) INCB16562, a JAK1/2 selective inhibitor, is efficacious against multiple myeloma cells and reverses the protective effects of cytokine and stromal cell support. Neoplasia 12(1):28–38
87. Hallek M, Neumann C, Schaffer M, Danhauser-Riedl S, von Bubnoff N, de Vos G et al (1997) Signal transduction of interleukin-6 involves tyrosine phosphorylation of multiple cytosolic proteins and activation of Src-family kinases Fyn, Hck, and Lyn in multiple myeloma cell lines. Exp Hematol 25(13):1367–1377
88. Ishikawa H, Tsuyama N, Abroun S, Liu S, Li FJ, Taniguchi O et al (2002) Requirements of src family kinase activity associated with CD45 for myeloma cell proliferation by interleukin-6. Blood 99(6):2172–2178
89. Staudt LM (2010) Oncogenic activation of NF-kappaB. Cold Spring Harb Perspect Biol 2(6):a000109
90. Landowski TH, Olashaw NE, Agrawal D, Dalton WS (2003) Cell adhesion-mediated drug resistance (CAM-DR) is associated with activation of NF-kappa B (RelB/p50) in myeloma cells. Oncogene 22(16):2417–2421
91. Demchenko YN, Glebov OK, Zingone A, Keats JJ, Bergsagel PL, Kuehl WM (2010) Classical and/or alternative NF-kappaB pathway activation in multiple myeloma. Blood 115(17): 3541–3552
92. Annunziata CM, Davis RE, Demchenko Y, Bellamy W, Gabrea A, Zhan F et al (2007) Frequent engagement of the classical and alternative NF-kappaB pathways by diverse genetic abnormalities in multiple myeloma. Cancer Cell 12(2):115–130
93. Keats JJ, Fonseca R, Chesi M, Schop R, Baker A, Chng WJ et al (2007) Promiscuous mutations activate the noncanonical NF-kappaB pathway in multiple myeloma. Cancer Cell 12(2):131–144
94. Yarde DN, Oliveira V, Mathews L, Wang X, Villagra A, Boulware D et al (2009) Targeting the Fanconi anemia/BRCA pathway circumvents drug resistance in multiple myeloma. Cancer Res 69(24):9367–9375
95. Tancred TM, Belch AR, Reiman T, Pilarski LM, Kirshner J (2009) Altered expression of fibronectin and collagens I and IV in multiple myeloma and monoclonal gammopathy of undetermined significance. J Histochem Cytochem 57(3):239–247

96. Hecht M, Heider U, Kaiser M, von Metzler I, Sterz J, Sezer O (2007) Osteoblasts promote migration and invasion of myeloma cells through upregulation of matrix metalloproteinases, urokinase plasminogen activator, hepatocyte growth factor and activation of p38 MAPK. Br J Haematol 138(4):446–458
97. Hehlgans S, Haase M, Cordes N (2007) Signalling via integrins: implications for cell survival and anticancer strategies. Biochim Biophys Acta 1775(1):163–180
98. Schmidmaier R, Morsdorf K, Baumann P, Emmerich B, Meinhardt G (2006) Evidence for cell adhesion-mediated drug resistance of multiple myeloma cells in vivo. Int J Biol Markers 21(4):218–222
99. Ohwada C, Nakaseko C, Koizumi M, Takeuchi M, Ozawa S, Naito M et al (2008) CD44 and hyaluronan engagement promotes dexamethasone resistance in human myeloma cells. Eur J Haematol 80(3):245–250
100. Nefedova Y, Sullivan DM, Bolick SC, Dalton WS, Gabrilovich DI (2008) Inhibition of Notch signaling induces apoptosis of myeloma cells and enhances sensitivity to chemotherapy. Blood 111(4):2220–2229
101. Hazlehurst LA, Dalton WS (2001) Mechanisms associated with cell adhesion mediated drug resistance (CAM-DR) in hematopoietic malignancies. Cancer Metastasis Rev 20(1–2): 43–50
102. Hazlehurst LA, Landowski TH, Dalton WS (2003) Role of the tumor microenvironment in mediating de novo resistance to drugs and physiological mediators of cell death. Oncogene 22(47):7396–7402
103. Turner JG, Marchion DC, Dawson JL, Emmons MF, Hazlehurst LA, Washausen P et al (2009) Human multiple myeloma cells are sensitized to topoisomerase II inhibitors by CRM1 inhibition. Cancer Res 69(17):6899–6905
104. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS (1999) Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood 93(5):1658–1667
105. Damiano JS, Hazlehurst LA, Dalton WS (2001) Cell adhesion-mediated drug resistance (CAM-DR) protects the K562 chronic myelogenous leukemia cell line from apoptosis induced by BCR/ABL inhibition, cytotoxic drugs, and gamma-irradiation. Leukemia 15(8):1232–1239
106. Hazlehurst LA, Valkov N, Wisner L, Storey JA, Boulware D, Sullivan DM et al (2001) Reduction in drug-induced DNA double-strand breaks associated with beta1 integrin-mediated adhesion correlates with drug resistance in U937 cells. Blood 98(6):1897–1903
107. Neri P, Ren L, Azab AK, Brentnall M, Gratton K, Klimowicz AC et al (2011) Integrin {beta}7-mediated regulation of multiple myeloma cell adhesion, migration, and invasion. Blood 117(23):6202–6213
108. Hazlehurst LA, Argilagos RF, Dalton WS (2007) Beta1 integrin mediated adhesion increases Bim protein degradation and contributes to drug resistance in leukaemia cells. Br J Haematol 136(2):269–275
109. Kazi A, Lawrence H, Guida WC, McLaughlin ML, Springett GM, Berndt N et al (2009) Discovery of a novel proteasome inhibitor selective for cancer cells over non-transformed cells. Cell Cycle 8(12):1940–1951
110. Schilling G, Hansen T, Shimoni A, Zabelina T, Perez-Simon JA, Gutierrez NC et al (2008) Impact of genetic abnormalities on survival after allogeneic hematopoietic stem cell transplantation in multiple myeloma. Leukemia 22(6):1250–1255
111. Emmons M, Boulware D, Sullivan DM, Hazlehurst LA (2006) Topoisomerase II beta levels are a determinant of melphalan-induced DNA crosslinks and sensitivity to cell death. Biochem Pharmacol 72(1):11–18
112. Turner JG, Gump JL, Zhang C, Cook JM, Marchion D, Hazlehurst L et al (2006) ABCG2 expression, function, and promoter methylation in human multiple myeloma. Blood 108(12): 3881–3889
113. Hazlehurst LA, Damiano JS, Buyuksal I, Pledger WJ, Dalton WS (2000) Adhesion to fibronectin via beta1 integrins regulates p27kip1 levels and contributes to cell adhesion mediated drug resistance (CAM-DR). Oncogene 19(38):4319–4327

114. Lwin T, Hazlehurst LA, Dessureault S, Lai R, Bai W, Sotomayor E et al (2007) Cell adhesion induces p27Kip1-associated cell-cycle arrest through down-regulation of the SCFSkp2 ubiquitin ligase pathway in mantle-cell and other non-Hodgkin B-cell lymphomas. Blood 110(5):1631–1638
115. Shain KH, Landowski TH, Dalton WS (2002) Adhesion-mediated intracellular redistribution of c-Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein-long confers resistance to CD95-induced apoptosis in hematopoietic cancer cell lines. J Immunol 168(5):2544–2553
116. Nefedova Y, Cheng P, Alsina M, Dalton WS, Gabrilovich DI (2004) Involvement of Notch-1 signaling in bone marrow stroma-mediated de novo drug resistance of myeloma and other malignant lymphoid cell lines. Blood 103(9):3503–3510
117. Matsui W, Wang Q, Barber JP, Brennan S, Smith BD, Borrello I et al (2008) Clonogenic multiple myeloma progenitors, stem cell properties, and drug resistance. Cancer Res 68(1):190–197
118. Meads MB, Hazlehurst LA, Dalton WS (2008) The bone marrow microenvironment as a tumor sanctuary and contributor to drug resistance. Clin Cancer Res 14(9):2519–2526
119. Lentzsch S, Gries M, Janz M, Bargou R, Dorken B, Mapara MY (2003) Macrophage inflammatory protein 1-alpha (MIP-1 alpha) triggers migration and signaling cascades mediating survival and proliferation in multiple myeloma (MM) cells. Blood 101(9):3568–3573
120. Nefedova Y, Landowski TH, Dalton WS (2003) Bone marrow stromal-derived soluble factors and direct cell contact contribute to de novo drug resistance of myeloma cells by distinct mechanisms. Leukemia 17(6):1175–1182
121. Mitsiades N, Mitsiades CS, Poulaki V, Chauhan D, Fanourakis G, Gu X et al (2002) Molecular sequelae of proteasome inhibition in human multiple myeloma cells. Proc Natl Acad Sci USA 99(22):14374–14379
122. Prince HM, Honemann D, Spencer A, Rizzieri DA, Stadtmauer EA, Roberts AW et al (2009) Vascular endothelial growth factor inhibition is not an effective therapeutic strategy for relapsed or refractory multiple myeloma: a phase 2 study of pazopanib (GW786034). Blood 113(19):4819–4820
123. Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R et al (1999) Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity 10(1):105–115
124. Tassone P, Neri P, Burger R, Savino R, Shammas M, Catley L et al (2005) Combination therapy with interleukin-6 receptor superantagonist Sant7 and dexamethasone induces antitumor effects in a novel SCID-hu in vivo model of human multiple myeloma. Clin Cancer Res 11(11):4251–4258
125. Rossi J-F, Manges RF, Voorhees P, Sutherland HJ, Orlowski RZ (2008) Preliminary results of CNTO 328, an anti-IL-6 monoclonal antibody, in combination with bortezomib in the treatment of relapsed or refractory multiple myeloma. Blood 112:867
126. Devine SM, Flomenberg N, Vesole DH, Liesveld J, Weisdorf D, Badel K et al (2004) Rapid mobilization of CD34+ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-Hodgkin's lymphoma. J Clin Oncol 22(6):1095–1102
127. Tavor S, Eisenbach M, Jacob-Hirsch J, Golan T, Petit I, Benzion K et al (2008) The CXCR4 antagonist AMD3100 impairs survival of human AML cells and induces their differentiation. Leukemia 22(12):2151–5158
128. Wong D, Korz W (2008) Translating an Antagonist of Chemokine Receptor CXCR4: from bench to bedside. Clin Cancer Res 14(24):7975–7980
129. Bisping G, Wenning D, Kropff M, Gustavus D, Muller-Tidow C, Stelljes M et al (2009) Bortezomib, dexamethasone, and fibroblast growth factor receptor 3-specific tyrosine kinase inhibitor in t(4;14) myeloma. Clin Cancer Res 15(2):520–531
130. Ramakrishnan V, Timm M, Haug JL, Kimlinger TK, Wellik LE, Witzig TE et al (2010) Sorafenib, a dual Raf kinase/vascular endothelial growth factor receptor inhibitor has significant anti-myeloma activity and synergizes with common anti-myeloma drugs. Oncogene 29(8):1190–1202

131. Matsunaga T, Takemoto N, Sato T, Takimoto R, Tanaka I, Fujimi A et al (2003) Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. Nat Med 9(9):1158–1165
132. Ricart AD, Tolcher AW, Liu G, Holen K, Schwartz G, Albertini M et al (2008) Volociximab, a chimeric monoclonal antibody that specifically binds alpha5beta1 integrin: a phase I, pharmacokinetic, and biological correlative study. Clin Cancer Res 14(23):7924–7929
133. Kimura RH, Levin AM, Cochran FV, Cochran JR (2009) Engineered cystine knot peptides that bind alphavbeta3, alphavbeta5, and alpha5beta1 integrins with low-nanomolar affinity. Proteins 77(2):359–369
134. Nair RR, Emmons MF, Cress AE, Argilagos RF, Lam K, Kerr WT et al (2009) HYD1-induced increase in reactive oxygen species leads to autophagy and necrotic cell death in multiple myeloma cells. Mol Cancer Ther 8(8):2441–2451
135. Pedranzini L, Dechow T, Berishaj M, Comenzo R, Zhou P, Azare J et al (2006) Pyridone 6, a pan-Janus-activated kinase inhibitor, induces growth inhibition of multiple myeloma cells. Cancer Res 66(19):9714–9721
136. Sun CY, Hu Y, Huang J, Chu ZB, Zhang L, She XM et al (2010) Brain-derived neurotrophic factor induces proliferation, migration, and VEGF secretion in human multiple myeloma cells via activation of MEK-ERK and PI3K/AKT signaling. Tumour Biol 31(2):121–128
137. Coluccia AM, Cirulli T, Neri P, Mangieri D, Colanardi MC, Gnoni A et al (2008) Validation of PDGFRbeta and c-Src tyrosine kinases as tumor/vessel targets in patients with multiple myeloma: preclinical efficacy of the novel, orally available inhibitor dasatinib. Blood 112(4):1346–1356

Chapter 9
Osteoclasts: Potential Target for Blocking Microenvironmental Support of Myeloma

Deborah L. Galson, Sonia D'Souza, and G. David Roodman

Abstract Multiple myeloma (MM) bone disease is a major contributor to the morbidity and mortality of MM patients due to pathological fractures. The MM cells interact with the cells of the bone microenvironment to both generate bone lesions as a result of enhanced induction of osteoclastogenesis and prevent reactive new bone formation to heal the lesions by repressing osteoblast activity. The MM stimulated osteoclasts (OCLs) not only generate bone lesions, but also interact with the myeloma cells to promote the proliferation and survival of the MM cells through the generation of interleukin-6 (IL-6), osteopontin, fibroblast activation protein, BAFF, APRIL, and annexin II. These MM-supportive OCL products present therapeutic opportunities. Further, the enhanced bone resorption by OCLs releases immobilized growth factors from the bone matrix that both support the MM cells and further stimulate OCL differentiation in a vicious cycle. Hence, targeting osteoclast activity may inhibit myeloma growth. Therefore, bisphosphonates have been investigated for their anti-tumor affects. The MM cells increase osteoclast activity both directly and by stimulation of microenvironmental production of RANKL, MIP-1α, TNF-α and interleukins IL-1β, IL-3 and IL-6. These are therefore also possible therapeutic targets to inhibit myeloma bone disease.

D.L. Galson • S. D'Souza
Department of Medicine, Division of Hematology/Oncology,
University of Pittsburgh, Pittsburgh, PA, USA

G.D. Roodman, M.D., Ph.D. (✉)
Division of Hematology Oncology,
Indian University Medical Center, Indianapolis IN
e-mail: roodmangd@upmc.edu

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_9,

9.1 Introduction

Multiple myeloma (MM) is the most common cancer to involve bone with more than 80% of patients developing bone lesions [1]. The bone lesions are purely osteolytic in nature and do not heal in the vast majority of patients, even when they are in long-term complete remission. Up to 20% of patients will present with a fracture at diagnosis, 40% will sustain a pathologic fracture within the first year of diagnosis, and 60% of patients will develop pathologic fractures over the course of their disease [2]. MM bone disease is so severe because MM, like other osteolytic metastases, has increased osteoclastic bone destruction, but in contrast to other tumors, once MM tumor burden exceeds 50% in a local area, osteoblast activity is either severely depressed or absent [3].

Bone destruction in MM can involve any bone and is responsible for some of the most devastating aspects of the disease. The most common radiographic findings of bone involvement in MM are "punched-out" lytic lesions without reactive new bone formation and also include osteopenia, pathologic fractures, or a combination of these conditions. These findings demonstrate that enhanced osteoclast (OCL) activity is a major contributor to MM bone disease, which is further exacerbated by the suppressed osteoblast activity. This paradigm makes the OCL an attractive target for treating MM bone disease.

9.2 Role of the Osteoclast in Myeloma

The bone marrow microenvironment plays a pivotal role in the development of MM bone disease. Multiple factors are produced by both the MM cells and neighboring bone marrow stromal cells (BMSC) within the microenvironment, which interact to shift the normal delicate balance of bone destruction and new bone formation toward increased bone destruction with absent new bone formation. In addition, the OCL themselves play an important role in supporting MM cell growth.

Yaccoby and coworkers have shown that primary MM plasma cells from patients are attracted to OCL precursors and that MM cells induce differentiation of these cells into multinucleated bone resorbing OCL [4]. They further showed that a coculture of MM cells with OCL allowed the primary MM cells to proliferate for more than 13 weeks. Physical contact between OCL and MM cells was required for these effects, and both OCL from healthy donors and MM patients could support the growth of MM cells. Blocking IL-6 decreased survival of MM cells but had no effect on the proliferation of the primary MM cells. Similarly, Abe and co-workers [5] have shown that OCL support the growth of primary MM cells and that this is dependent on both osteopontin and IL-6 production by the OCL. These authors demonstrated that peripheral blood mononuclear cell-derived OCL were much more potent in enhancing the growth and survival of primary MM cells than BMSC. They also showed that OCL protected MM cells from apoptosis induced by serum depletion or treatment of MM cells with doxorubicin. Again, adhesion of the MM cells to

OCL was required to support MM cell growth as complete inhibition of cell contact between MM cells and OCL totally blocked the supportive effects of OCL on MM cell growth. These data clearly showed that OCL play a pivotal role in the support of MM cell growth. The adhesive interactions between MM cells and OCL increased IL-6 production by OCL. Osteopontin (Opn) receptors, VLA-4, $\alpha_v\beta_3$-integrin, and CD44 are expressed on the cell surface of myeloma cells. IL-6 and Opn in combination enhanced MM cell growth and survival. However, other factors must also be involved in OCL supported MM cell growth, because it is only partially inhibited by simultaneous addition of anti-osteopontin and anti-IL-6 antibodies. As discussed further below, IL-6 has multiple sources and roles in MM bone disease; however, IL-6 production by OCLs may increase MM tumor burden leading to enhanced bone destruction.

Other OCL-derived factors have been implicated in the support of myeloma cells. Ge et al. found that the DASH protease, fibroblast activation protein (FAP), was involved in the OCL-induced MM cell growth [6]. These authors demonstrated that FAP was upregulated when OCL and MM cells were cocultured in vitro as well as in MM tissue in human bone in the SCID-hu model of MM. FAP was expressed by OCL and was critical for the support of MM cell growth by OCL. In addition, knockdown of FAP expression with a siRNA reduced MM cell survival in these cocultures. Inhibition of DASH proteases with PT-100 affected expression of adhesive molecules by OCL that are required for OCL support of MM cell growth and MM bone disease [7]. Further, inhibition of DASH proteases blocked OCL differentiation and bone resorption activity. Tanaka and coworkers [8] have shown that MM cell–OCL interactions enhance angiogenesis. These authors found that OCL-derived osteopontin and VEGF produced by MM cells cooperatively enhanced angiogenesis and induced osteoclastogenic activity by vascular endothelial cells. These data clearly show that the OCL plays a central role in both MM cell growth and the increased angiogenesis associated with MM. Further, Abe and coworkers reported that BAFF and APRIL are OCL-derived survival factors for MM cells [9], which are also produced by bone BMSC from myeloma patients. Thus, BAFF, produced by both OCL and BMSC in patients with MM, is a potential therapeutic target for treating MM bone disease.

Recently, we have found that OCL produce annexin II (AXII), which is a stimulator of MM cell growth [10], by both increasing proliferation and decreasing apoptosis [11] and is also an autocrine/paracrine stimulator of OCL formation [12, 13]. AXII was found to be upregulated in pancreatic, stomach, lung, renal, breast cancers, and more importantly in MM [14–20]. More recently, AXII was shown to increase the proliferation of human MM cell lines and had anti-apoptotic effects in these MM cell lines [11]. The AXII/AXII receptor (AXIIR) axis plays a crucial role in the homing, growth, and adhesion of prostate cancer cells to the bone marrow [21]. AXII appears to stimulate MM cell growth through increased ERK and p38 MAPK signaling. This is consistent with previous studies in which we have shown that AXII can also stimulate receptor activator of NF-κB ligand (RANKL) expression in human BMSC via MAPK as well as GM-CSF expression by both marrow stromal cells and activated T cells [10, 22]. RANKL and GM-CSF together are important

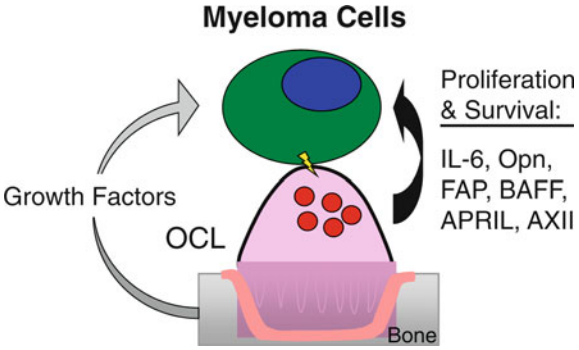


Fig. 9.1 *Mechanisms of osteoclastic support for myeloma cells*. Osteoclasts are stimulated by cell–cell contact with myeloma cells to produce a variety of factors that support myeloma proliferation and survival, such as IL-6, Opn, FAP, BAFF, APRIL, and AXII. The bone destructive process releases growth factors that increase the growth of myeloma cells and increase OCL progenitors, further exacerbating both processes

for OCL formation induced by AXII. MM cells themselves also make AXII, but it appears in preliminary studies that MM-derived AXII does not increase MM cell growth, whereas both OCL- and BMSC-derived AXII stimulate the growth of MM cells. Thus taken together, these data demonstrate a critical role for OCL in the support of MM cell tumor proliferation and prevention of MM cell apoptosis (Fig. 9.1).

9.3 Osteoclast Stimulatory Factors Produced in Myeloma

In addition to factors produced by OCLs, osteoclastic bone resorption releases growth factors, which enhance the growth of MM cells (Fig. 9.1). This has been termed the "vicious cycle" for MM cell growth in which MM cells induce increased OCL activity and the bone resorption process releases immobilized growth factors produced by the marrow microenvironment that both support the MM cells and further stimulate OCL. Locally acting factors produced by MM cells have been implicated in both the extensive bone destruction and impaired new bone formation. The factors produced in vivo by MM cells or induced by MM in bone microenvironmental cells that can increase osteoclastic activity include RANKL, macrophage inflammatory protein-1α (MIP-1α), TNF-α, IL-1β, IL-3, and IL-6 [23–27] (Fig. 9.2).

RANKL is part of the tumor necrosis factor (TNF) gene family and is a major osteoclastogenic factor involved in MM bone disease. When MM cells bind to BMSC, RANKL expression is increased on the surface of the BMSC. Subsequently, this results in enhanced OCL activity through binding of RANKL to its receptor RANK on OCL precursor cells, promoting their differentiation [28]. RANKL also plays a role in the inhibition of OCL apoptosis [29]. T-lymphocytes also produce RANKL in the MM marrow microenvironment. The proposed mechanism for the

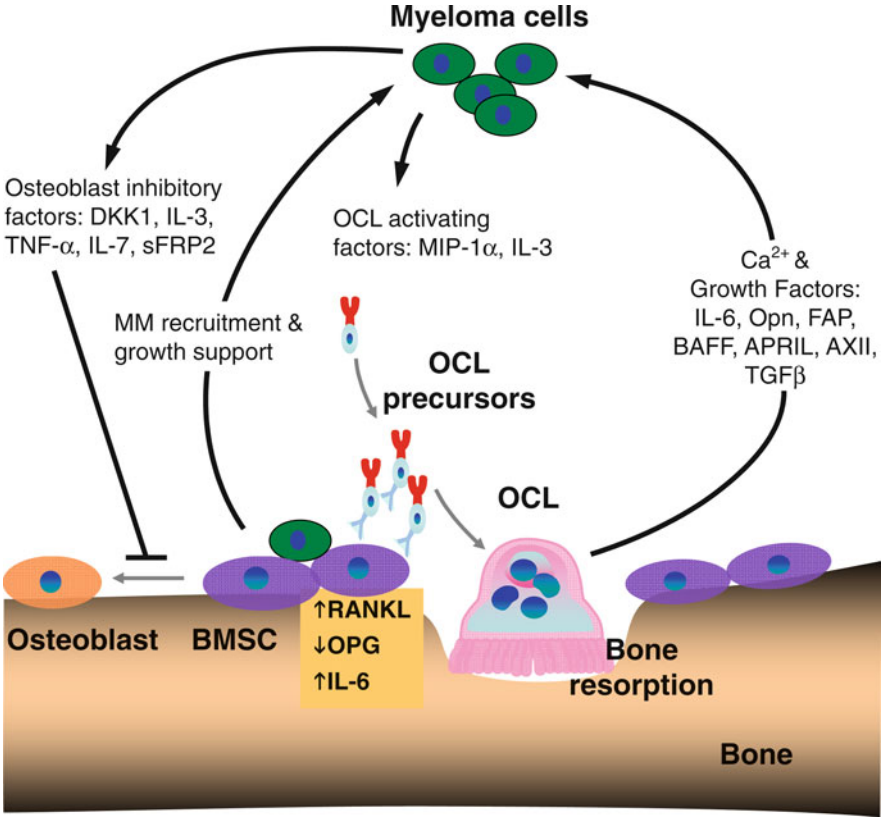


Fig. 9.2 *Mechanisms responsible for myeloma bone disease*. Myeloma cells produce factors that directly or indirectly activate osteoclasts such as MIP-1α, TNF-α, IL-1β, and IL-3. In addition, MM cells enhance osteoclast formation and activation by inducing BMSC production of IL-6 and altering the RANKL/OPG ratio. Myeloma cells also produce dickkopf-1 (DKK-1), IL-3, soluble frizzle-related protein-2 (sFRP2), TNF-α, and IL-7, which suppress osteoblast differentiation and new bone formation

upregulation is through the release of a soluble factor by MM cells, which increases RANKL expression on the T-lymphocytes and BMSC and ultimately results in enhanced osteolytic bone destruction [30].

A soluble decoy for RANKL, known as osteoprotegerin (OPG), is produced by BMSC and inhibits the actions of RANKL on osteoclastogenesis. The ratio of RANKL to OPG determines the level of OCL formation and activity. Interactions between MM cells and BMSC lead to decreased production of OPG, which allows for increased amounts of RANKL binding to its receptor. This results in further OCL activation and enhanced bone destruction [29]. Giuliani et al. have demonstrated that in cocultures of human MM cells with BMSC, RANKL expression was upregulated and OPG production strongly downregulated at both the protein and mRNA levels in the BMSC [25]. In addition, Pearse et al. have examined bone marrow

biopsy specimens from patients with MM and found that RANKL expression was markedly upregulated in bone marrow biopsies from patients with MM, while OPG was expressed at very low levels compared to normal controls [31]. The above studies suggest that there is a marked imbalance between RANKL expression and OPG levels that favors osteoclastogenesis and OCL activation in MM.

In a murine model of MM, Menu et al. demonstrated that injected Fc-OPG inhibited the development of MM-induced osteolytic bone disease and also led to a significant reduction in tumor load [32]. Similarly, when primary MM cells are injected into a human fetal bone rudiment implanted into mice with severe combined immunodeficiency (SCID), a RANKL inhibitor, RANK-Fc, decreased bone resorption and tumor burden [33]. These studies suggest that blocking bone resorption induced by RANKL may decrease tumor burden as well as bone destruction in patients with MM. Based on these observations, a human monoclonal antibody to RANKL has been developed and used in phase I, II, and III trials in MM patients, and is discussed below.

Recently, antagonists to the MIP-1α receptor, CCR1, have been developed, and tested in vitro and in vivo in preclinical models. These experiments have demonstrated their potential utility in treating MM bone disease. Oba and coworkers reported that the CCR1 antagonist, BX471, inhibited OCL formation induced by MIP-1α and blocked adhesion of MM cells to BMSC. This resulted in decreased secretion of IL-6 by the BMSC [34]. Similarly, Vallet et al., using another CCR1 antagonist MLN3897, showed that MLN3897 inhibited OCL formation and inhibited the adhesion of MM cells to OCL, thereby decreasing MM cell growth and survival [35]. Menu et al. have reported studies using the 5TMM mouse model of MM in which BX471 decreased development of osteolytic lesions by 40% in mice with established tumors [36]. Taken together, these results demonstrate that CCR1 is a viable target for treating MM bone disease and should be pursued. It is expected that CCR1 antagonists will be in clinical trial for MM in the next several years.

TNF-α and IL-1β induce IL-6 and RANKL production [37] and can also synergize with RANKL to potentiate OCL formation (TNF-α) [38] as well as OCL activation and survival (IL-1β) [39]. However, their source and roles in MM bone disease are unclear [23, 40]. In particular, a pilot study of recombinant human soluble TNF receptor fusion protein (Etanercept) in patients with refractory multiple myeloma did not result in an objective response. Furthermore, acceleration of disease occurred in four of ten patients [41]. In a phase II clinical trial with 47 patients with smoldering and indolent MM who were at risk of progression to active myeloma, treatment with IL-1 receptor antagonist (IL-1Ra) and low-dose dexamethasone was reported at ASCO 2007 to induce a chronic disease state with improved progression-free survival [42]. More recently, in preclinical studies, a humanized anti-IL-1β antibody (XOMA 052) was highly effective at inhibiting IL-6 production generated by all MM patient supernatants from bone marrow cells tested including the patients that were high inducers of paracrine IL-6 production.

IL-3 is also significantly elevated in marrow plasma from patients with MM as compared to normal controls [26]. Previous reports have shown that up to 40% of patients with MM will have elevated levels of IL-3 in the peripheral blood, and 75%

of bone marrow samples from patients with MM will have elevated IL-3 mRNA and protein levels [24]. Serum from MM patients with elevated IL-3 stimulates the growth of IL-3 dependent MM cell lines [43]. IL-3 can induce in vitro OCL formation in human marrow cultures at levels similar to those measured in MM patient samples, and OCL formation induced by marrow plasma from MM patients could be inhibited by a blocking antibody to IL-3 [26]. IL-3 also enhances the effects of RANKL and MIP-1α on the growth and development of OCLs, as well as directly stimulates MM cell growth [26]. Further, addition of IL-3 to murine bone marrow induces the development of OCL-like cells, which were multinucleated and stained positively for tartrate resistant acid-phosphatase (a marker enzyme of OCLs) [44]. Overall, IL-3 increases the numbers and activity of OCLs, leading to further bone destruction, and appears to be an OCL stimulatory factor in MM.

The role that IL-6 plays in MM is controversial. It is unclear if elevated levels of IL-6 correlate with disease status [24, 45]. Levels of IL-6 have been shown to be elevated in patients with osteolytic lesions, as compared to patients without lytic lesions or with patients with monoclonal gammopathy of undetermined significance (MGUS) [46]. IL-6 levels from bone marrow, but not peripheral blood plasma, have also been correlated with markers of bone turnover [47]. IL-6 induces RANKL expression in mesenchymal cells thereby increasing osteoclastogenesis [48, 49]. Most studies support the idea that IL-6 is produced by cells in the bone marrow microenvironment induced through contact with MM cells. These cell types include osteoblasts, OCLs, and BMSC. Increased osteoblast production of IL-6 has been reported in cocultures of human osteoblasts with MM cells [50]. OCLs also produce high levels of IL-6 when grown in coculture with MM cells [5]. The increased IL-6 not only increases OCL formation but also further enhances the growth of the MM cells and inhibits MM cell apoptosis [5, 51]. Based on these observations, humanized monoclonal antibodies to both IL-6 and IL-6R have been developed and will be discussed below.

Because multiple signaling pathways are activated in BMSC from MM patients, that regulate both BMSC support of MM and induction of osteoclast formation, attempts have been made to try to identify a common component that is involved in these multiple signaling pathways and can be targeted to treat MM bone disease. BMSC from MM patients have increased NF-κB and p38 MAPK signaling. p62 is a common component that serves as a platform for formation of these signaling complexes. However, the effects of targeting p62 on these signaling pathways in MM are unknown. We found that although p62 levels were not altered in the BMSC of 13 MM patients compared to 11 healthy controls, signaling through p62 was increased in BMSC from MM patients compared with healthy cells as exemplified by elevated ratios of phosho-PKCζ to total PKCζ (two to sixfold), although the levels varied greatly among the individual patients. Therefore, we determined the effects of siRNA knockdown of p62 in BMSC on p38 MAPK and NF-κB signaling. p62 expression was decreased by 60% and 90% at the mRNA and protein level, respectively, in these BMSC. PKC and VCAM-1 expressions were decreased by at least 70% in p62 siRNA transduced MM-derived and normal BMSC compared with control siRNA transduced cells. Further, knocking-down p62 in primary MM-derived

BMSC treated with TNF-α markedly decreased NF-κB and p38 MAPK signaling compared with control siRNA treated cells. Importantly, IL-6 production by p62 siRNA transfected normal and MM-derived BMSC was also significantly decreased compared with scrambled siRNA or untreated cells. We further showed that loss of p62 markedly decreased the capacity of MM patient-derived BMSC to both induce OCL formation and enhance the growth of MM cells. These results demonstrate that targeting p62 may be a method for blocking the role of the microenvironment in MM bone disease.

9.4 Targeting Osteoclast Generation and Activity to Inhibit Tumor Growth in Myeloma

9.4.1 Bisphosphonates

Nitrogen-containing bisphosphonates interfere with OCL function and survival and have been extensively utilized to treat osteoporosis [52]. These compounds bid avidly to the surface of bone hydroxyapatite crystals and are ingested by OCL during bone resorption. These drugs interfere with metabolic pathways involving diphosphate moieties such as the mevalonate pathway involved in cholesterol synthesis and prenylation of GTPases Rab, Rho, and Ras. This leads to disturbance of the OCL cytoskeleton resulting in decreased bone resorption and increased OCL apoptosis.

9.5 Anti-Myeloma Effects of Bisphosphonates in Preclinical Models of Myeloma

Studies in preclinical models of MM and bone metastases [53–56] demonstrated that bisphosphonates inhibit tumor growth and decrease bone destruction in vivo. Yaccoby and coworkers reported that pamidronate and zoledronate decreased tumor growth in a SCID-hu model of MM [57]. In this model, human fetal bone is implanted subcutaneously in mice with severe combined immunodeficiency. Primary human MM cells are then injected into the fetal bone. The MM cells grow in this human microenvironment and induce bone resorption. Treatment of these mice with pamidronate or zoledronate inhibited MM-induced bone resorption and MM cell growth, if MM cells were from patients with disease confined to the bone marrow. In contrast, pamidronate and zoledronate did not inhibit tumor growth when MM cells from patients with extramedullary disease were used. These results suggested that the anti-MM effects of bisphosphonates only occurred if the MM cells were dependent on the marrow microenvironment and/or bone resorption for growth.

Similarly, Croucher et al. used the 5T2MM model of MM to test the effects of bisphosphonates on MM growth and bone destruction [58]. The 5T2MM model of MM is an immunocompetent model of MM in which murine MM cells derived from a spontaneously developing MM in mice are injected intravenously into syngeneic hosts. The mice develop a disease that has all the characteristics of human MM. Zoledronate treatment, either from time of tumor injection or after paraprotein was detected, prevented osteolytic lesions, decreased tumor burden, and significantly increased survival of the mice from 35 to 47 days after detection of the paraprotein. Zoledronate also blocked the increased angiogenesis induced by the MM cells. These results suggest that bisphosphonates inhibit tumor-induced angiogenesis through their effects on MM cells and/or on endothelial cells. Radl et al. [59] reported that pamidronate also reduced tumor burden and increased survival in the 5TMM2 model of MM.

However, bisphosphonates also significantly reduce the growth of prostate, lung, and breast cancer cells implanted subcutaneously in mice (reviewed in [60]), suggesting that bisphosphonates can also inhibit tumor growth independent of their effects on bone remodeling. Bisphosphonates can also directly inhibit growth, induce apoptosis, and increase sensitivity to chemotherapy in MM cell lines. Guenther and coworkers [56] reported that zoledronate inhibited the growth of six different MM cell lines. Importantly, the concentrations of zoledronate required to induce cytotoxicity in MM cells did not affect peripheral blood mononuclear cells from healthy donors. Baulch-Brown and coworkers also showed that zoledronate inhibited MM cell growth and that the inhibitory effects of zoledronate on MM cell growth were due to its capacity to prevent geranylgeranylation of small GTPases that resulted in cell cycle arrest and apoptosis [61]. Bisphosphonates also inhibit MM cell adhesion to BMSC [62], increasing the sensitivity of MM cells to chemotherapy [63]. Since small GTPases play a key role in integrin activation, the inhibition of tumor cell adhesion to matrix or BMSC by bisphosphonates is not surprising [64]. Further, zoledronate inhibits chemokine-induced tumor cell migration by affecting cell surface expression of the chemokine receptor CXCR4, a receptor for CXCL12 [65]. CXCR4 and CXCL12 play important roles in MM cell homing to the marrow and MM cell mobilization to the peripheral blood [66]. Finally, zoledronate can synergize with several chemotherapeutic agents, to increase tumor cell apoptosis and enhance TNF-α related apoptosis through TRAIL [66, 67]. These in vitro results demonstrate the direct anti-MM potential of bisphosphonates.

9.6 Clinical Studies Reporting Effects of Bisphosphonates in Treatment of Myeloma

The seminal studies of Berenson and coworkers [68] demonstrated that pamidronate significantly increased the time to development and decreased the number of skeletal related events (SREs) as well as bone pain in patients with advanced MM. However, pamidronate did not significantly increase survival of these patients.

Attal and coworkers examined the efficacy of pamidronate as maintenance therapy for MM patients after autologous stem cell transplantation [69]. Six hundred patients were randomly assigned to receive no maintenance, pamidronate, or pamidronate with thalidomide following autologous stem cell transplantation. None of the patients received pamidronate prior to transplantation. Pamidronate did not decrease SREs or increase 3-year event-free or overall survival in the patients. In contrast, both event-free survival and overall survival were significantly increased in patients receiving pamidronate with thalidomide. These results demonstrated that pamidronate as a single agent did not confer a survival advantage in patients with MM. However, this trial could not distinguish if pamidronate enhanced the effects of thalidomide on event-free and overall survival because no patients received thalidomide without pamidronate in the trial agent. Anecdotally, Kondo and coworkers reported an MM patient treated for 18 months with pamidronate and no additional anti-MM treatment [70]. Pamidronate markedly reduced marrow plasmacytosis and sbin levels in this patient.

Several studies have shown that bisphosphonates have antitumor effects in breast cancer patients when used in the adjuvant setting. Diel and coworkers and Powles et al. reported that treatment of patients with primary breast cancer at high risk for distant metastasis with clodronate decreased bone metastasis and increased overall survival compared to placebo [71, 72]. Visceral metastases also decreased in patients treated with adjuvant clodronate [73]. Gnant et al. recently reported that treatment of premenopausal breast cancer patients with endocrine therapy and zoledronate improved disease-free survival as well as decreased bone and distant metastasis but did not improve overall survival [74]. Further, large trials of zoledronate for prevention of treatment-induced bone loss in premenopausal breast cancer patients receiving aromatase inhibitors or postmenopausal patients receiving adjuvant endocrine therapy for stages 1 to 3A hormone responsive breast cancer found a significant decrease in both bone and distant metastasis as well as increased disease-free survival (reviewed in [75]). In addition, patients receiving neoadjuvant chemotherapy and zoledronate had an increased complete remission rate as well as decreased residual tumor size at surgery [75]. These results suggest that zoledronate may have antitumor effects in breast cancer patients independent of its effects on bone.

Until recently, a distinct survival advantage for zoledronate treatment of patients with MM has not been reported [76]. Avilés et al. treated 94 newly diagnosed MM patients with conventional chemotherapy and either zoledronate or placebo [77]. Five-year actuarial event-free survival and overall survival was increased for patients receiving zoledronate compared to controls (80% vs. 46%, $p<0.01$). However, this trial did not determine if the effects of zoledronate on survival were independent of zoledronate's effects on SREs. However, at the 2010 American Society of Clinical Oncology Meeting, Morgan and colleagues reported the results of the MRC Myeloma IX trial [78]. This was a prospective multicenter randomized controlled trial comparing intravenous zoledronate (4 mg every 3–4 weeks) with daily oral clodronate in patients randomized to either intensive therapy, which included stem cell transplantation, or less intensive therapy. MM treatment was

dependant on the performance status of the patient. Almost 2,000 newly diagnosed MM patients were entered into this trial. Patients had international staging system (ISS) stage I, II, or III MM. Approximately 20% of the patients did not have bone disease. At a median follow-up of 3.7 years, SREs were significantly reduced in patients treated with zoledronate as compared to clodronate (27% vs. 35%, $p=0.0004$). Importantly, patients treated with zoledronate had a 5.5-month survival advantage compared to those receiving clodronate. Zoledronate treatment decreased the risk of death by 16% and progress-free survival by 12% ($p=0.0118$ and $p=0.0179$, respectively). Multivariate analysis demonstrated that this survival advantage was independent of zoledronate's effects on SREs. The incidence of osteonecrosis of the jaw was low in the study (3.6% vs. 0.3% in zoledronate vs. clodronate-treated patients). Further, Dr. Morgan stated at the presentation that patients who do not have bone disease and received zoledronate also had a similar survival advantage compared to clodronate.

How zoledronate enhanced the survival of MM patients in this large prospective randomized trial is unclear. Zoledronate could affect patient survival through its effects on OCL, or it may have direct effects on MM cells. OCL are angiogenic cells [79], and zoledronate's inhibition of OCL activity may contribute to decreased angiogenesis in MM patients. Another potential mechanism for the enhanced disease-free survival of MM patients receiving zoledronate could be prevention of MM cell mobilization to distant bone marrow sites. Kollet and colleagues reported that OCL play a role in hematopoietic stem cell mobilization through degradation of CXCL12 [80]. The CXCR4/CXCL12 axis also contributes to mobilization of MM cells from the bone marrow of patients with MM [81]. Thus, blocking OCL activity should inhibit MM cell mobilization. However, patients without bone disease had the same survival advantage as those with bone disease, and multivariate analysis found that the survival advantage was independent of SREs. Nitrogen-containing bisphosphonates can have immunomodulatory effects and stimulate expansion of γΔ-T cells, thereby increasing tumor cell lysis by γΔ-T cells [82]. Zoledronate could also affect tumor growth through its effects on endothelial cells, angiogenesis, and decreasing VEGF production, as demonstrated in patients with metastatic breast cancer [78]. Thus, the effects of zoledronate on patient survival cannot be completely explained by its inhibition of OCL activity. However, because of the complexity of this trial, further analysis will be required to determine the mechanism(s) responsible for the survival advantage conferred by zoledronate in MM patients.

9.6.1 RANKL Inhibition as a Target to Inhibit Tumor Growth in Myeloma

Preclinical and clinical studies clearly identified the importance of RANKL as a driver of osteoclastogenesis in MM, and several studies have suggested that MM cells themselves can produce RANKL as well as induce BMSC and activated T cell

RANKL production [83, 84]. Importantly, preclinical studies using OPG have shown that blocking RANKL activity markedly decreases bone destruction and tumor burden in murine models of MM [85, 86]. These studies have led to the development of a high infinity human monoclonal antibody that binds RANKL, denosumab. Denosumab specifically binds RANKL and does not bind other gene family members such as TNF-α, TNF-β, TRAIL, or CD40 [87]. It directly inhibits OCL formation and activation as well as affects OCL survival. Phase I studies have shown that denosumab at 1–3 mg/kg given subcutaneously as a single dose can suppress bone resorption markers for up to 90 days. The suppression of bone resorption markers induced by denosumab was at the same level as that seen with a single dose of 90 mg of pamidronate. However, pamidronate suppression of bone resorption markers only lasted about 30 days [88]. A phase II study of denosumab in patients with relapsed and plateau phase MM showed that denosumab was very effective for MM bone disease with bone resorption markers decreased in relapsed patients by 70% and a 52% decrease in bone resorption markers in plateau phase patients [89]. Recently, results of a phase III trial that compared denosumab to zoledronic acid in MM in patients with solid tumor bone metastasis, but not breast cancer or prostate cancer, have been reported [90]. Denosumab was noninferior in delaying or preventing the first on study skeletal-related event compared to zoledronic acid in over 1,600 patients, of which approximately 200 were MM patients. Further, adverse event rates with denosumab and zoledronic acid were similar, and the incidence of ONJ was infrequent and not significantly different between the treatment arms (10 vs. 11 patients). Thus, denosumab is equally efficacious as zoledronic acid in patients with MM although it is unclear what the long-term effects of denosumab will be because of the small number of MM patients in the phase III study.

9.6.2 Blocking IL-6 to Treat Myeloma Bone Disease

Both IL-6 and IL-6R (gp80) have been targeted through the development of humanized mAbs (reviewed in [91, 92]). Anti-IL-6 antibodies developed by Diaclone (B-E8) and Centocor (CNTO 328) have been used alone or in combination with chemotherapeutic agents in preclinical studies and in small phase I clinical studies of MM. Both B-E8 (half-life 3–4 days) and CNTO 328 (half-life 18 days) transiently blocked IL-6 action, decreased C-reactive protein production, generated antiproliferative effects, and decreased IL-6 toxic effects such as fever and were well tolerated. It is not clear why the therapeutic effects of both anti-IL-6 antibodies were transient. The anti-IL6R mAb (Tocilizumab/Actemra®) is already in use for treatment of Castleman's disease and rheumatoid arthritis (specifically blocking inflammatory osteoclastogenesis) and has shown effectiveness for juvenile idiopathic arthritis and Crohn's disease. However, phase I/II clinical trials in MM have just begun.

9.7 Summary

The OCL appears to play a critical role in supporting the growth of MM cells, both by the direct effects of factors produced by OCL, including BAFF, APRIL, osteopontin, IL-6, and AXII. In addition, the bone destructive process ongoing in MM cells releases growth factors, which stimulate the growth of MM cells from the bone microenvironment. Targeting OCL activity in MM with bisphosphonates appears to improve survival of MM patients and suggests that combinations of therapies that target both OCL activity and the tumor cells themselves should have a profound effect on MM bone disease and MM tumor growth in general. Future studies with denosumab will determine if it too has anti-MM effects comparable to those recently reported with zoledronic acid.

References

1. Roodman GD (2004) Pathogenesis of myeloma bone disease. Blood Cells Mol Dis 32:290–292
2. Melton LJ 3rd, Kyle RA, Achenbach SJ et al (2005) Fracture risk with multiple myeloma: a population-based study. J Bone Miner Res 20:487–493
3. Taube T, Beneton MN, McCloskey EV et al (1992) Abnormal bone remodelling in patients with myelomatosis and normal biochemical indices of bone resorption. Eur J Haematol 49:192–198
4. Yaccoby S, Wezeman MJ, Henderson A et al (2004) Cancer and the microenvironment: myeloma-osteoclast interactions as a model. Cancer Res 64:2016–2023
5. Abe M, Hiura K, Wilde J et al (2004) Osteoclasts enhance myeloma cell growth and survival via cell–cell contact: a vicious cycle between bone destruction and myeloma expansion. Blood 104:2484–2491
6. Ge Y, Zhan F, Barlogie B et al (2006) Fibroblast activation protein (FAP) is upregulated in myelomatous bone and supports myeloma cell survival. Br J Haematol 133:83–92. doi:10.1111/j.1365-2141.2006.05976.x
7. Pennisi A, Li X, Ling W et al (2009) Inhibitor of DASH proteases affects expression of adhesion molecules in osteoclasts and reduces myeloma growth and bone disease. Br J Haematol 145:775–787. doi:10.1111/j.1365-2141.2009.07696.x
8. Tanaka Y, Abe M, Hiasa M et al (2007) Myeloma cell–osteoclast interaction enhances angiogenesis together with bone resorption: a role for vascular endothelial cell growth factor and osteopontin. Clin Cancer Res 13:816–823. doi:10.1158/1078-0432.CCR-06-2258
9. Abe M, Kido S, Hiasa M et al (2006) BAFF and APRIL as osteoclast-derived survival factors for myeloma cells: a rationale for TACI-Fc treatment in patients with multiple myeloma. Leukemia 20:1313–1315. doi:10.1038/sj.leu.2404228
10. D'Souza S, Shiozawa Y, Galson DL et al (2009) Annexin II and Annexin II receptor interactions enhance multiple myeloma growth in the bone marrow microenvironment. In: The IX international meeting on cancer induced bone disease, Arlington, VA (Abstract)
11. Bao H, Jiang M, Zhu M et al (2009) Overexpression of Annexin II affects the proliferation, apoptosis, invasion and production of proangiogenic factors in multiple myeloma. Int J Hematol 90:177–185. doi:10.1007/s12185-009-0356-8
12. Takahashi S, Reddy SV, Chirgwin JM et al (1994) Cloning and identification of annexin II as an autocrine/paracrine factor that increases osteoclast formation and bone resorption. J Biol Chem 269:28696–28701

13. Menaa C, Devlin RD, Reddy SV et al (1999) Annexin II increases osteoclast formation by stimulating the proliferation of osteoclast precursors in human marrow cultures. J Clin Invest 103:1605–1613
14. Cole SP, Pinkoski MJ, Bhardwaj G et al (1992) Elevated expression of annexin II (lipocortin II, p36) in a multidrug resistant small cell lung cancer cell line. Br J Cancer 65:498–502
15. Shinar DM, Schmidt A, Halperin D et al (1993) Expression of alpha v and beta 3 integrin subunits in rat osteoclasts in situ. J Bone Miner Res 8:403–414
16. Vishwanatha JK, Chiang Y, Kumble KD et al (1993) Enhanced expression of annexin II in human pancreatic carcinoma cells and primary pancreatic cancers. Carcinogenesis 14:2575–2579
17. Emoto K, Sawada H, Yamada Y et al (2001) Annexin II overexpression is correlated with poor prognosis in human gastric carcinoma. Anticancer Res 21:1339–1345
18. Claudio JO, Masih-Khan E, Tang H et al (2002) A molecular compendium of genes expressed in multiple myeloma. Blood 100:2175–2186. doi:10.1182/blood-2002-01-0008
19. Zimmermann U, Woenckhaus C, Pietschmann S et al (2004) Expression of annexin II in conventional renal cell carcinoma is correlated with Fuhrman grade and clinical outcome. Virchows Arch 445:368–374. doi:10.1007/s00428-004-1103-4
20. Sharma MR, Koltowski L, Ownbey RT et al (2006) Angiogenesis-associated protein annexin II in breast cancer: selective expression in invasive breast cancer and contribution to tumor invasion and progression. Exp Mol Pathol 81:146–156. doi:10.1016/j.yexmp.2006.03.003
21. Shiozawa Y, Havens AM, Jung Y et al (2008) Annexin II/Annexin II receptor axis regulates adhesion, migration, homing, and growth of prostate cancer. J Cell Biochem 370:370–380. doi:10.1002/jcb.21835
22. Li F, Chung H, Reddy SV et al (2005) Annexin II stimulates RANKL expression through MAPK. J Bone Miner Res 20:1161–1167
23. Costes V, Portier M, Lu ZY et al (1998) Interleukin-1 in multiple myeloma: producer cells and their role in the control of IL-6 production. Br J Haematol 103:1152–1160
24. Choi SJ, Cruz JC, Craig F et al (2000) Macrophage inflammatory protein 1-alpha is a potential osteoclast stimulatory factor in multiple myeloma. Blood 96:671–675
25. Giuliani N, Colla S, Rizzoli V (2004) New insight in the mechanism of osteoclast activation and formation in multiple myeloma: focus on the receptor activator of NF-kappaB ligand (RANKL). Exp Hematol 32:685–691
26. Lee JW, Chung HY, Ehrlich LA et al (2004) IL-3 expression by myeloma cells increases both osteoclast formation and growth of myeloma cells. Blood 103:2308–2315
27. Gunn WG, Conley A, Deininger L et al (2006) A crosstalk between myeloma cells and marrow stromal cells stimulates production of DKK1 and interleukin-6: a potential role in the development of lytic bone disease and tumor progression in multiple myeloma. Stem Cells 24:986–991
28. Ehrlich LA, Roodman GD (2005) The role of immune cells and inflammatory cytokines in Paget's disease and multiple myeloma. Immunol Rev 208:252–266
29. Sezer O, Heider U, Jakob C et al (2002) Immunocytochemistry reveals RANKL expression of myeloma cells. Blood 99:4646–4647
30. Giuliani N, Colla S, Sala R et al (2002) Human myeloma cells stimulate the receptor activator of nuclear factor-kappa B ligand (RANKL) in T lymphocytes: a potential role in multiple myeloma bone disease. Blood 100:4615–4621
31. Pearse RN, Sordillo EM, Yaccoby S et al (2001) Multiple myeloma disrupts the TRANCE/osteoprotegerin cytokine axis to trigger bone destruction and promote tumor progression. Proc Natl Acad Sci USA 98:11581–11586
32. Menu E, Asosingh K, Van Riet I et al (2004) Myeloma cells (5TMM) and their interactions with the marrow microenvironment. Blood Cells Mol Dis 33:111–119. doi:10.1016/j.bcmd.2004.04.012
33. Epstein J, Yaccoby S (2005) The SCID-hu myeloma model. Methods Mol Med 113:183–190. doi:10.1385/1-59259-916-8:183
34. Oba Y, Lee JW, Ehrlich LA et al (2005) MIP-1alpha utilizes both CCR1 and CCR5 to induce osteoclast formation and increase adhesion of myeloma cells to marrow stromal cells. Exp Hematol 33:272–278

35. Vallet S, Raje N, Ishitsuka K et al (2007) MLN3897, a novel CCR1 inhibitor, impairs osteoclastogenesis and inhibits the interaction of multiple myeloma cells and osteoclasts. Blood 110:3744–3752. doi:10.1182/blood-2007-05-093294
36. Menu E, De Leenheer E, De Raeve H et al (2006) Role of CCR1 and CCR5 in homing and growth of multiple myeloma and in the development of osteolytic lesions: a study in the 5TMM model. Clin Exp Metastasis 23:291–300. doi:10.1007/s10585-006-9038-6
37. Wei S, Kitaura H, Zhou P et al (2005) IL-1 mediates TNF-induced osteoclastogenesis. J Clin Invest 115:282–290. doi:10.1172/JCI23394
38. Lam J, Takeshita S, Barker JE et al (2000) TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. J Clin Invest 106:1481–1488. doi:10.1172/JCI11176
39. Nakamura I, Jimi E (2006) Regulation of osteoclast differentiation and function by interleukin-1. Vitam Horm 74:357–370. doi:10.1016/S0083-6729(06)74015-8
40. Sati HI, Greaves M, Apperley JF et al (1999) Expression of interleukin-1beta and tumour necrosis factor-alpha in plasma cells from patients with multiple myeloma. Br J Haematol 104:350–357
41. Tsimberidou AM, Waddelow T, Kantarjian HM et al (2003) Pilot study of recombinant human soluble tumor necrosis factor (TNF) receptor (p75) fusion protein (TNFR:Fc; Enbrel) in patients with refractory multiple myeloma: increase in plasma TNF alpha levels during treatment. Leuk Res 27:375–380. doi:S0145212602000826 [pii]
42. Lust JA, Lacy MQ, Zeldenrust SR et al (2009) Induction of a chronic disease state in patients with smoldering or indolent multiple myeloma by targeting interleukin 1{beta}-induced interleukin 6 production and the myeloma proliferative component. Mayo Clin Proc 84:114–122. doi:10.4065/84.2.114
43. Merico F, Bergui L, Gregoretti MG et al (1993) Cytokines involved in the progression of multiple myeloma. Clin Exp Immunol 92:27–31
44. Barton BE, Mayer R (1989) IL-3 induces differentiation of bone marrow precursor cells to osteoclast-like cells. J Immunol 143:3211–3216
45. Solary E, Guiguet M, Zeller V et al (1992) Radioimmunoassay for the measurement of serum IL-6 and its correlation with tumour cell mass parameters in multiple myeloma. Am J Hematol 39:163–171
46. Sati HI, Apperley JF, Greaves M et al (1998) Interleukin-6 is expressed by plasma cells from patients with multiple myeloma and monoclonal gammopathy of undetermined significance. Br J Haematol 101:287–295
47. Abildgaard N, Glerup H, Rungby J et al (2000) Biochemical markers of bone metabolism reflect osteoclastic and osteoblastic activity in multiple myeloma. Eur J Haematol 64:121–129
48. Roodman GD, Kurihara N, Ohsaki Y et al (1992) Interleukin 6. A potential autocrine/paracrine factor in Paget's disease of bone J Clin Invest 89:46–52
49. Palmqvist P, Persson E, Conaway HH et al (2002) IL-6, leukemia inhibitory factor, and oncostatin M stimulate bone resorption and regulate the expression of receptor activator of NF-kappa B ligand, osteoprotegerin, and receptor activator of NF-kappa B in mouse calvariae. J Immunol 169:3353–3362
50. Karadag A, Oyajobi BO, Apperley JF et al (2000) Human myeloma cells promote the production of interleukin 6 by primary human osteoblasts. Br J Haematol 108:383–390
51. Anderson KC, Jones RM, Morimoto C et al (1989) Response patterns of purified myeloma cells to hematopoietic growth factors. Blood 73:1915–1924
52. Russell RG, Watts NB, Ebetino FH et al (2008) Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. Osteoporos Int 19:733–759. doi:10.1007/s00198-007-0540-8
53. Corey E, Brown LG, Quinn JE et al (2003) Zoledronic acid exhibits inhibitory effects on osteoblastic and osteolytic metastases of prostate cancer. Clin Cancer Res 9:295–306
54. Hiraga T, Williams PJ, Ueda A et al (2004) Zoledronic acid inhibits visceral metastases in the 4 T1/luc mouse breast cancer model. Clin Cancer Res 10:4559–4567. doi:10.1158/1078-0432.CCR-03-0325

55. Koto K, Horie N, Kimura S et al (2009) Clinically relevant dose of zoledronic acid inhibits spontaneous lung metastasis in a murine osteosarcoma model. Cancer Lett 274:271–278. doi:10.1016/j.canlet.2008.09.026
56. Guenther A, Gordon S, Tiemann M et al (2010) The bisphosphonate zoledronic acid has antimyeloma activity in vivo by inhibition of protein prenylation. Int J Cancer 126:239–246. doi:10.1002/ijc.24758
57. Yaccoby S, Pearse RN, Johnson CL et al (2002) Myeloma interacts with the bone marrow microenvironment to induce osteoclastogenesis and is dependent on osteoclast activity. Br J Haematol 116:278–290
58. Croucher PI, De Hendrik R, Perry MJ et al (2003) Zoledronic acid treatment of 5T2MM-bearing mice inhibits the development of myeloma bone disease: evidence for decreased osteolysis, tumor burden and angiogenesis, and increased survival. J Bone Miner Res 18:482–492
59. Radl J, Croese JW, Zurcher C et al (1985) Influence of treatment with APD-bisphosphonate on the bone lesions in the mouse 5 T2 multiple myeloma. Cancer 55:1030–1040
60. Brown HK, Holen I (2009) Anti-tumour effects of bisphosphonates–what have we learned from in vivo models? Curr Cancer Drug Targets 9:807–823
61. Baulch-Brown C, Molloy TJ, Yeh SL et al (2007) Inhibitors of the mevalonate pathway as potential therapeutic agents in multiple myeloma. Leuk Res 31:341–352. doi:10.1016/j.leukres.2006.07.018
62. Corso A, Ferretti E, Lunghi M et al (2005) Zoledronic acid down-regulates adhesion molecules of bone marrow stromal cells in multiple myeloma: a possible mechanism for its antitumor effect. Cancer 104:118–125. doi:10.1002/cncr.21104
63. Nefedova Y, Landowski TH, Dalton WS (2003) Bone marrow stromal-derived soluble factors and direct cell contact contribute to de novo drug resistance of myeloma cells by distinct mechanisms. Leukemia 17:1175–1182. doi:10.1038/sj.leu.2402924
64. Azab AK, Azab F, Blotta S et al (2009) RhoA and Rac1 GTPases play major and differential roles in stromal cell-derived factor-1-induced cell adhesion and chemotaxis in multiple myeloma. Blood 114:619–629. doi:10.1182/blood-2009-01-199281
65. Denoyelle C, Hong L, Vannier JP et al (2003) New insights into the actions of bisphosphonate zoledronic acid in breast cancer cells by dual RhoA-dependent and -independent effects. Br J Cancer 88:1631–1640. doi:10.1038/sj.bjc.6600925
66. Karabulut B, Erten C, Gul MK et al (2009) Docetaxel/zoledronic acid combination triggers apoptosis synergistically through downregulating antiapoptotic Bcl-2 protein level in hormone-refractory prostate cancer cells. Cell Biol Int 33:239–246. doi:10.1016/j.cellbi.2008.11.011
67. Rachner TD, Singh SK, Schoppet M et al (2010) Zoledronic acid induces apoptosis and changes the TRAIL/OPG ratio in breast cancer cells. Cancer Lett 287:109–116. doi:10.1016/j.canlet.2009.06.003
68. Berenson JR, Lichtenstein A, Porter L et al (1996) Efficacy of pamidronate in reducing skeletal events in patients with advanced multiple myeloma. Myeloma Aredia Study Group N Engl J Med 334:488–493
69. Attal M, Harousseau JL, Leyvraz S et al (2006) Maintenance therapy with thalidomide improves survival in patients with multiple myeloma. Blood 108:3289–3294. doi:10.1182/blood-2006-05-022962
70. Kondo H, Mori A (2002) Anti-tumor activity of pamidronate in human multiple myeloma. Leuk Lymphoma 43:919–921
71. Powles T, Paterson S, Kanis JA et al (2002) Randomized, placebo-controlled trial of clodronate in patients with primary operable breast cancer. J Clin Oncol 20:3219–3224
72. Diel IJ, Jaschke A, Solomayer EF et al (2008) Adjuvant oral clodronate improves the overall survival of primary breast cancer patients with micrometastases to the bone marrow: a long-term follow-up. Ann Oncol 19:2007–2011. doi:10.1093/annonc/mdn429
73. Diel IJ, Solomayer EF, Costa SD et al (1998) Reduction in new metastases in breast cancer with adjuvant clodronate treatment. N Engl J Med 339:357–363
74. Gnant M, Mlineritsch B, Schippinger W et al (2009) Endocrine therapy plus zoledronic acid in premenopausal breast cancer. N Engl J Med 360:679–691. doi:10.1056/NEJMoa0806285

75. Lipton A (2010) Should bisphosphonates be utilized in the adjuvant setting for breast cancer? Breast Cancer Res Treat 122(3):627–636
76. Mhaskar R, Redzepovic J, Wheatley K et al. (2010) Bisphosphonates in multiple myeloma. Cochrane Database Syst Rev 3:CD003188. doi:10.1002/14651858.CD003188.pub2
77. Aviles A, Nambo MJ, Neri N et al (2007) Antitumor effect of zoledronic acid in previously untreated patients with multiple myeloma. Med Oncol 24:227–230. doi:MO:24:2:227 [pii]
78. Vincenzi B, Santini D, Dicuonzo G et al (2005) Zoledronic acid-related angiogenesis modifications and survival in advanced breast cancer patients. J Interferon Cytokine Res 25:144–151. doi:10.1089/jir.2005.25.144
79. Cackowski FC, Anderson JL, Patrene KD et al (2010) Osteoclasts are important for bone angiogenesis. Blood 115(1):140–149
80. Kollet O, Dar A, Shivtiel S et al (2006) Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. Nat Med 12:657–664. doi:10.1038/nm1417
81. Azab AK, Runnels JM, Pitsillides C et al (2009) CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy. Blood 113:4341–4351. doi:10.1182/blood-2008-10-186668
82. Caccamo N, Meraviglia S, Scarpa F et al (2008) Aminobisphosphonate-activated gammadelta T cells in immunotherapy of cancer: doubts no more. Expert Opin Biol Ther 8:875–883. doi:10.1517/14712598.8.7.875
83. Roodman GD (2002) Role of the bone marrow microenvironment in multiple myeloma. J Bone Miner Res 17:1921–1925
84. Sezer O, Heider U, Zavrski I et al (2003) RANK ligand and osteoprotegerin in myeloma bone disease. Blood 101:2094–2098
85. Doran PM, Turner RT, Chen D et al (2004) Native osteoprotegerin gene transfer inhibits the development of murine osteolytic bone disease induced by tumor xenografts. Exp Hematol 32:351–359. doi:10.1016/j.exphem.2004.01.006
86. Rabin N, Kyriakou C, Coulton L et al (2007) A new xenograft model of myeloma bone disease demonstrating the efficacy of human mesenchymal stem cells expressing osteoprotegerin by lentiviral gene transfer. Leukemia 21:2181–2191. doi:10.1038/sj.leu.2404814
87. Hamdy NA (2008) Denosumab: RANKL inhibition in the management of bone loss. Drugs Today (Barc) 44:7–21. doi:10.1358/dot.2008.44.1.1178467
88. Body JJ, Facon T, Coleman RE et al (2006) A study of the biological receptor activator of nuclear factor-kappaB ligand inhibitor, denosumab, in patients with multiple myeloma or bone metastases from breast cancer. Clin Cancer Res 12:1221–1228
89. Vij R, Horvath N, Spencer A et al (2009) An open-label, phase 2 trial of denosumab in the treatment of relapsed or plateau-phase multiple myeloma. Am J Hematol 84:650–656. doi:10.1002/ajh.21509
90. Henry D, von Moos R, Vadhan-Raj S et al (2009) A double-blind, randomized study of denosumab versus zoledronic acid for the treatment of bone metastases in patients with advanced cancer (excluding breast and prostate cancer) or multiple myeloma. Eur J Cancer Suppl 7:12 Abstract.
91. Munshi N, Tai Y-T (2008) Antibody and other immune-based therapies for myeloma. In: Lonial S (ed) Myeloma therapy: pursuing the plasma cell. Humana Press, Totowa, NJ. doi:10.1007/978-1-59745-564-0
92. Ara T, Declerck YA (2010) Interleukin-6 in bone metastasis and cancer progression. Eur J Cancer 46:1223–1231. doi:10.1016/j.ejca.2010.02.026

Chapter 10
Targeting the BAFF/APRIL Cytokine Network in Multiple Myeloma

Stephen A. Mihalcik and Diane F. Jelinek

Abstract The BAFF/APRIL cytokine network is intimately linked through three different receptors to the survival and fitness of B lineage cells, from the first expression of a complete B cell receptor to their differentiation to memory B and plasma cells. The specific, pervasive, and survival-linked nature of the relationship between B lineage cells and this cytokine network make it both a likely disease modifier and a tantalizing target for therapeutic intervention in humoral immune pathologies. Some current therapeutics directly targeting the BAFF/APRIL cytokine network have been developed and undergone clinical trials in the context of autoimmunity with some limited success. Despite a powerful rationale and a constantly deepening mechanistic understanding of the BAFF/APRIL cytokine network in normal and malignant plasma cells, trials of cytokine network-targeted therapeutics in multiple myeloma are still in their infancy and have shown only minor promise. There is significantly greater potential in inhibiting NF-κB, a downstream mediator of BAFF/APRIL signals.

10.1 Introduction

B cell activating factor of the TNF family (BAFF; also BLyS, TNFSF13B) is the most prominent member of a complex cytokine network central to both the homeostasis and pathology of humoral immunity. The network is defined by

S.A. Mihalcik
Department of Immunology, Mayo Graduate School, Mayo Clinic,
Rochester, MN 55905, USA

D.F. Jelinek, Ph.D. (✉)
Department of Immunology, Guggenheim 4, Mayo Clinic, College of Medicine,
200 First Street Southwest, Rochester, MN 55905, USA
e-mail: jelinek.diane@mayo.edu

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_10,

ligands BAFF and a proliferation inducing ligand (APRIL) and receptors BAFF-receptor (BAFF-R; also BR3, TNFRSF13C), B cell maturation antigen (BCMA; also TNFRSF17), and transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI; also TNFRSF13B). All three receptors are expressed primarily on B lineage cells and are differentially regulated at various stages of ontogeny and differentiation. Furthermore, appropriate function of these receptors has been shown to regulate B cell survival at crucial points in development. The qualities of lineage restriction, stage-specific differential expression, and survival enhancement make the BAFF network a tantalizing target for malignancies that arise from the B lineage. However, the potential for therapeutic intervention is exquisitely dependent on the still incompletely understood physiology of the system and currently ranges from very encouraging to the less than promising.

10.2 The BAFF Receptor/Ligand Network: Structure, Expression, and Specific Interactions

10.2.1 BAFF and APRIL

The two main ligands of the system (Fig. 1.1), BAFF [1–6] and APRIL [7, 8], are Type II transmembrane proteins with a receptor-binding tumor necrosis factor (TNF) homology domain (THD) at the carboxyl terminus separated from the amino terminal transmembrane domain by a furin cleavage site. In addition to these two predominant, canonical members of the network, there are also multiple BAFF and APRIL splice variants [9]. The most prominent of these variants are ΔBAFF [10], which lacks an exon encoding part of the THD, and TWE-PRIL [11], which is the product of a *trans*-splicing event combining the 5′end of the 5′ adjacent gene *TWEAK* (TNF-related weak inducer of apoptosis) with the 3′end of APRIL. Although ΔBAFF retains the exon encoding full-length BAFF's furin cleavage site, it is less susceptible to cleavage and has been shown to dramatically reduce endogenous BAFF activity by becoming incorporated into BAFF trimers and thus blocking the release of soluble BAFF and also by lessening the soluble trimer's bioactivity [10, 12]. While this is an area of active research, little is known about the in vivo physiological significance of any of the BAFF and APRIL splice variants.

BAFF is present both in a membrane-bound form on the surface of the cells that produce it and in a soluble form predominantly as a homotrimer [3, 13, 14]. The furin cleavage of APRIL, however, occurs intracellularly, and extracellular APRIL is only detectable in its soluble form [15–18]. There is evidence that both BAFF and APRIL are capable of acting physiologically as ligands of higher order than trimers. Unique among the TNF family, BAFF has been proposed to self-assemble into a 60-mer viral-like particle using a flap region to associate each

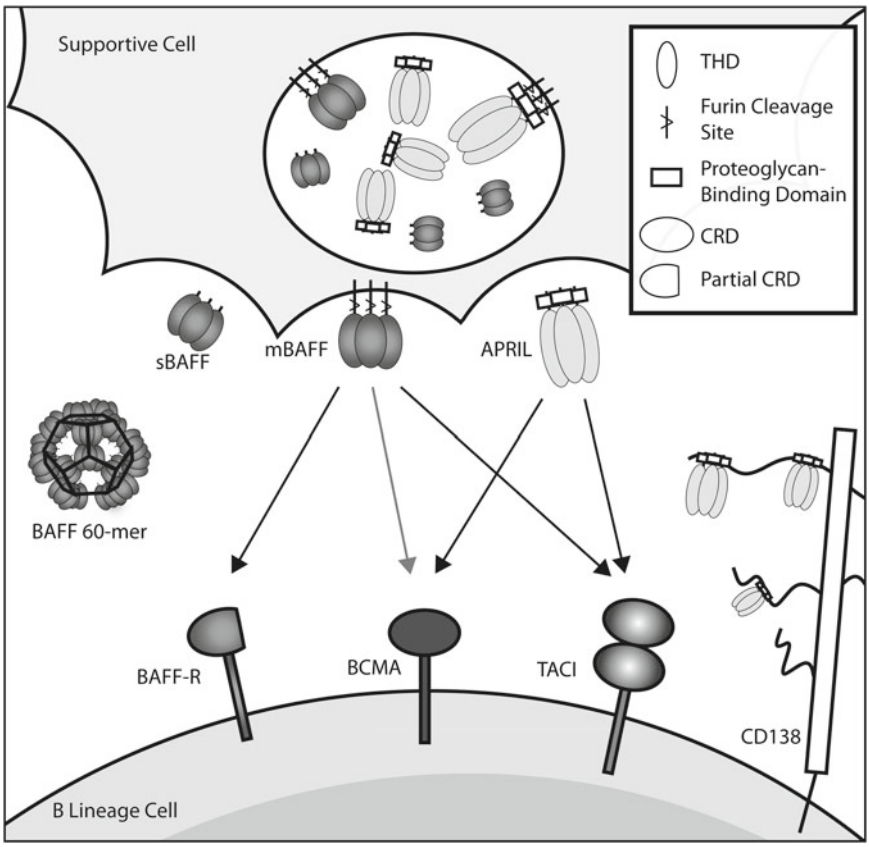


Fig. 1.1 *The BAFF/APRIL cytokine network.* The schematic represents the two main ligands of the network, BAFF and APRIL, as produced by supportive cells of the immune system, including dendritic cells, macrophages, osteoclasts, and stromal cells. BAFF is present in the immune milieu both as a soluble trimer (sBAFF), a membrane-bound trimer (mBAFF), and a viral-like particle 60-mer, while APRIL is present only in soluble form or bound to HSPGs. Heteromers of the ligands and BAFF and APRIL variants are not represented. BAFF-R, BCMA, and TACI are present at different points in B lineage development and differentiation, and the ligands of the system have varying affinity for each receptor. BAFF-R only binds BAFF, TACI binds both APRIL and BAFF with comparable affinity, and BCMA has a distinctly higher affinity for APRIL than BAFF. THD, TNF homology domain; *CRD* cysteine-rich domain

of the 20 trimers [19–21] though the physiological relevance of the 60-mer is still a matter of debate [22–24]. The existence of the BAFF 60-mer is especially pertinent to a discussion of BAFF-targeted therapeutics, since this form of the BAFF ligand would have distinct biological properties that affect its localization, pharmacokinetics, and activity, including a 20-fold increase in avidity and a likely size-imposed inability to diffuse across endothelial barriers. While APRIL does not assemble into viral-like complexes, it has been shown to effectively create a higher order ligand by binding heparin sulfate proteoglycans like syndecans

including CD138 on the surface of cells, allowing the accumulation of APRIL trimers at high concentration in the immediate vicinity of their target receptors [18, 25, 26]. These complexities in the form of higher order BAFF and APRIL ligands represent a significant barrier to targeted therapeutics since such therapeutic strategies must overcome the enhanced avidity of multimerized molecules and also reach the compartments to which the multimerization restricts them: the microenvironment in which the BAFF 60-mer assembles and the surface of HSPG- and BBR-coexpressing target cells.

BAFF and APRIL have been shown to have numerous sources throughout the innate immune system, including neutrophils, monocytes, macrophages, and dendritic cells [1, 3, 27–30] as well as a class of radiation-resistant stromal cells [31]. These sources likely provide the bulk of these factors, although both BAFF and APRIL have been shown repeatedly to have potentially autocrine sources, as well, expressed by both malignant, and in rare cases, normal B lineage cells that co-express their receptors [32–36]. While the antiapoptotic loop of autocrine BAFF and APRIL production is intriguing, the relative contribution of this alternative pathway to BBR signaling in malignancy is undefined. Autocrine pathways could present an especially difficult challenge to therapies targeting either the ligands or the receptors of this cytokine network. Since cells engaging an autocrine loop may not require the surface expression of the receptors or the secretion of the ligands, both normally extracellular arms of the system would be rendered inaccessible to most therapeutics.

10.2.2 BAFF-R, TACI, and BCMA

BAFF-R [37, 38], TACI [39–43], and BCMA [44–46] are members of the TNF receptor (TNFR) family. Their ligand-binding regions are defined by the presence of cysteine-rich domains (CRDs) that allow binding to the THDs of their ligand counterparts. The initial descriptions and structural taxonomy of these receptors were complicated by their atypical structure; while most TNFRs contain three or more CRDs, TACI has only two, BCMA has only one, and BAFF-R has only a single, partial CRD. Despite this structural deviation, it is now clear that the extracellular domains of these receptors do indeed bind the THD of their ligand counterparts. The intracellular domains of these molecules are characterized by TNFR associated factor (TRAF) binding. Specifically, BCMA has been shown to bind TRAFs 1, 2, and 3; TACI to bind TRAFs 2, 5, and 6; and BAFF-R to specifically bind only TRAF3 [41, 45, 47–49].

All three of these specific receptors bind BAFF to varying degrees [9]. BAFF binding to BAFF-R and TACI is both easily demonstrable and several orders of magnitude greater than BAFF binding to BCMA. Unlike BAFF, APRIL does not bind BAFF-R and binds only to TACI and BCMA. APRIL has an additional non-TNFR binding partner in heparan sulfate proteoglycans (HSPGs), including syndecans like CD138 [18, 25]. Several basic residues distinct from APRIL's specific

receptor binding site impart an ability to bind HSPGs while simultaneously binding its specific TNFR partners [50, 51]. This additional binding ability grants APRIL the capacity to collect and multimerize on the surface of cells with surface HSPGs, cells that may either possess specific APRIL receptors like plasma cells, or like bone marrow stromal cells, that interact with cells that do. This additional HSPG-binding function is particularly adaptive to TACI function, since TACI signaling seems to be dependent on higher-order ligands of the type that could be provided endogenously by HSPG-multimerized APRIL, the BAFF-R 60-mer, or membrane-bound BAFF-R [24]. Additionally, APRIL-binding HSPGs may initiate a distinct signal [52] and also provide a unique sink for circulating APRIL, amplifying its effect by concentrating it on the surface of target cells and allowing otherwise insignificant amounts of paracrine or autocrine APRIL to provide a viable signal.

In humans, the BAFF and APRIL receptors are largely differentially restricted to B lineage cells at various stages of development and differentiation (Fig. 1.2) [18, 53–56]. BAFF-R is first detectably expressed on the surface of B cells at the immature stage, when B cells first express a complete and functional BCR, increases in mature B cells, and is present at various levels on all B cells before the loss of expression in terminally differentiated bone marrow plasma cells. TACI expression follows a more inducible phenotype; it is first expressed on a small subset of naïve B cells and a larger portion of activated B cells and clearly characterizes memory B cells and plasma cells. BCMA is the defining BAFF/APRIL receptor of terminally differentiated plasma cells, although there are also germinal center and memory B subpopulations with some degree of BCMA expression.

10.3 BAFF and APRIL Receptors in Plasma Cells

10.3.1 Normal Plasma Cells

There is accumulating evidence that BCMA is the defining BBR of plasma cells. The increasingly well-defined BBR profile of B lineage cells demonstrates both an induction of BCMA coincident to loss of BAFF-R upon differentiation to immunoglobulin secreting cells and the consistent presence of BCMA on bone marrow PCs [54]. While the absence or improper signaling of BAFF-R and TACI have been shown to have significant effects on developing B cell populations [9], they have had little effect on the independent physiology of their downstream descendants, plasma cells. However, the BCMA-mediated pathway has been shown to have significant ramifications for the long-lived plasma cell subset; its absence in effect eliminates the longevity of those cells [57] and may impact the survival of plasmablasts as well [58]. Recent work has demonstrated that this survival pathway can rely on either BAFF or APRIL, both of which are capable of binding BCMA [59], although the multimerization that CD138 provides may shift the physiologic responsibility of BCMA signaling to APRIL in vivo.

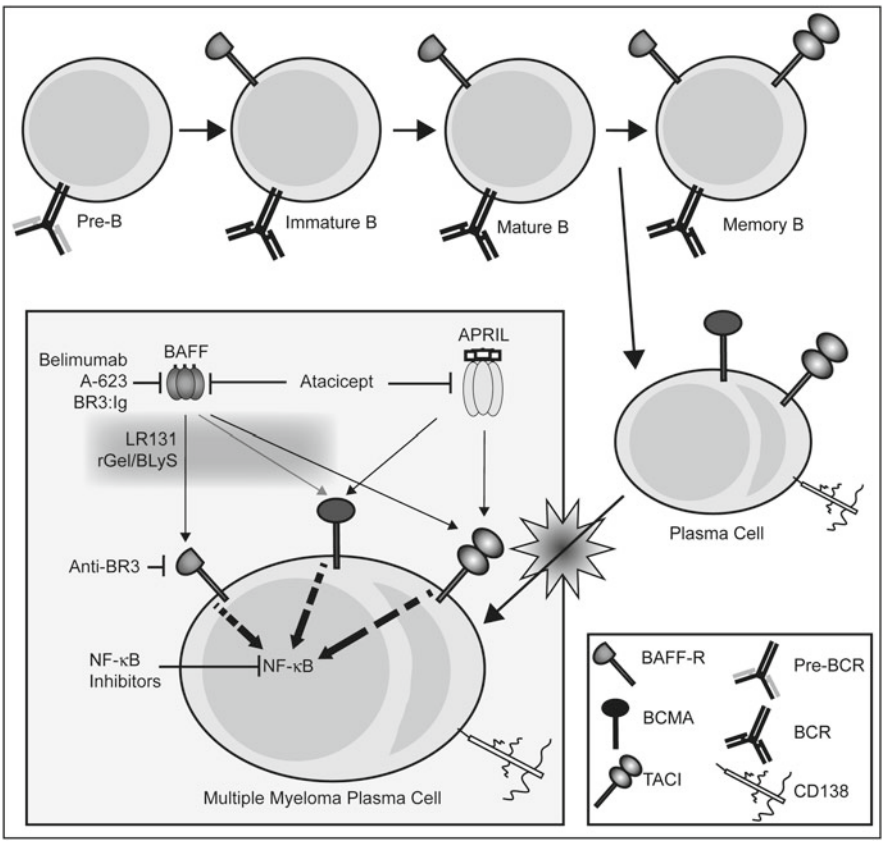


Fig. 1.2 *The BAFF/APRIL network in physiological development, differentiation, and in plasma cell malignancy.* While BAFF-R is expressed in all B cells from the first expression of a complete BCR until differentiation to a plasma cell, TACI is induced only at later stages of development beginning at activation, and BCMA identifies plasma cells almost exclusively. *Inset.* In multiple myeloma, the BBR profile mostly recapitulates the expression profile of their normal counterparts, although some primary myeloma cells have been shown to express surface BAFF-R. Therapeutic agents targeting the BAFF/APRIL cytokine network aim at the ligands, the receptors, and at NF-κB, a central, but by no means sole, mediator of signaling downstream of the three receptors

10.3.2 Myeloma Plasma Cells

Despite the mounting consensus regarding BCMA's role in normal plasma cell physiology and the diminished role of BAFF-R and TACI, the BAFF-binding receptor profile of these cells' malignant counterparts is still somewhat ambiguous. There is agreement that BCMA is expressed by the malignant plasma cells that characterize multiple myeloma, but TACI and BAFF-R expression have been shown to vary among both primary patient samples and established multiple myeloma cell lines [35, 60]. This characterization was most recently reinforced by

a gene expression profiling study of 320 newly diagnosed myeloma patients [61] whose data we analyzed to show universally high-level BCMA expression, variable and significantly lower absolute TACI expression, and low to absent BAFF-R expression. While BAFF-R expression in myeloma has been largely dismissed as insignificant due to its only sporadic presence on primary myeloma cells and conspicuous absence on normal bone marrow plasma cells, the repeated segregation of primary myeloma cells and myeloma cell lines into either $TACI^{hi}$ or $TACI^{lo}$ groups has inspired significant exploration. Moreaux et al. have postulated that the $TACI^{hi}$ and $TACI^{lo}$ groups respectively represent a mature bone marrow, niche-dependent myeloma subtype and a plasmablastic, niche-independent myeloma subtype [62, 63]. More importantly, there is evidence that these BAFF-binding receptors can enhance proliferation and survival of myeloma cells [35, 60, 64, 65] and that the TACI signature affects this response [26, 66].

10.4 Targeting BAFF in Multiple Myeloma

10.4.1 Targeting the BAFF Network in B Lineage Malignancies

Although all B lineage malignancies have shown some degree of BBR expression, the central role of the BAFF/BAFF-R axis in the survival of normal developing B cells has made BAFF and BAFF-R attractive targets in cancers characterized by a BAFF-rich milieu or that express BAFF-R. In mice, BAFF and APRIL transgenic animals demonstrate significant expansions of B cells and, in certain circumstances, predispositions to B lineage neoplasms [67–69]. In B cell non-Hodgkins lymphoma and chronic lymphocytic leukemia, in which BBR expression is well documented, exogenous BAFF has been shown to support the malignant cells and BAFF levels have been shown to correlate with disease progression and severity [32, 34, 70–75]. Thus, there is a powerful rationale behind targeting the BAFF/APRIL cytokine network in malignancy, although the BAFF/BAFF-R axis in mature B cells has been the most obvious and exploited arm in this approach.

10.4.2 Points of Intervention in BAFF/APRIL Network Targeting in Multiple Myeloma

The rationale for BAFF/APRIL network-targeted intervention in multiple myeloma is not as compelling as that for the BAFF-R-expressing B lineage malignancies, which express the most well-characterized and survival-linked BBR. However, it is clear that normal bone marrow plasma cells and malignant myeloma cells express BCMA, can express TACI albeit at low and variable levels, and, in some circumstances, express BAFF-R. Furthermore, the ability of the malignant cells to exploit BAFF- and APRIL-initiated signaling pathways has

been repeatedly demonstrated with in vitro assays [35, 60, 64–66]. The evidence implicating the BAFF/APRIL network in the regulation of multiple myeloma growth and survival suggests three significant points of intervention to target multiple myeloma (1) the presence of ligands BAFF and APRIL in the immune milieu, (2) the receptors characteristic of the malignant myeloma cells, and (3) the signaling cascades downstream of the BBRs responsible for mediating the BAFF- and APRIL-initiated effects. The state of therapeutics specifically targeting BAFF, APRIL, and their receptors (Fig. 1.2) in autoimmunity and oncology was recently reviewed in detail by Ryan et al. [76].

10.4.2.1 Ligand-Targeted Therapy

Therapeutics targeting the ligands of the BAFF/APRIL network are the most well-developed class of BAFF/APRIL network-directed drugs. This class includes molecules that have already had some degree of testing in humans and, in the case of atacicept, testing in myeloma.

Atacicept (TACI:Fc5), a modified fusion protein combining the APRIL- and BAFF-binding domain of the TACI molecule with the Fc portion of human immunoglobulin IgG1, was designed to act as a decoy receptor, preventing BAFF and APRIL from meeting their endogenous binding partners by providing an excess of a soluble alternative and thus depleting free soluble BAFF and APRIL stores. In vitro investigations provided the proof-of-principle evidence that atacicept could act through APRIL/BAFF depletion to inhibit tumor growth [60, 64, 66], while a recently completed phase I trial showed some promise in both Waldenström's macroglobulinemia and multiple myeloma. In this study, atacicept showed little toxicity and measurable clinical and biological effects that corresponded with stable, though not dramatically improved, disease in most subjects [77, 78]. Specifically, "five of the [eleven] patients with [multiple myeloma] who completed the first treatment cycle had stable disease, and four of these maintained stable disease after the extension treatment period," meaning that disease metrics including M-protein level, urinary light chain excretion, and bone marrow plasma cells did not change more than 25% and thus, according to established criteria [79], these patients did not experience a minimal response or progression. Besides trials in plasma cell malignancies, atacicept is undergoing extensive studies in antibody-driven autoimmune disorders and in non-Hodgkin's lymphoma [80–85]. These trials support the favorable toxicity profile and biological efficacy of atacicept. As these studies progress, it will be important to address the distribution of atacicept, since intervening in the BAFF/APRIL network in plasma cell malignancies may mean accessing the bone marrow and, in advanced disease, lytic lesions, which may be difficult to access, and interrupting the bone marrow microenvironment and the intimate relationship between plasma cells and the adjacent supportive cells.

In addition to atacicept, which binds both BAFF and APRIL, there are several nascent pharmaceuticals targeting BAFF alone, including belimumab, an antibody to BAFF; A-623 (formerly AMG-623), a synthetic BAFF antagonist; and BR3-Ig, a

BAFF-R-immunoglobulin fusion protein. The most developed of this class is belimumab, a fully human monoclonal antibody directed against BAFF, which is also known by the trade name Lymphostat-B©, developed by Human Genome Sciences and GlaxoSmithKline. Like atacicept, all the members of this class of therapeutics are proposed to work by depletion of the target ligand, rendering it unavailable to ligate the three receptors of the network. Unlike atacicept, they are incapable of removing APRIL from the system. APRIL is particularly relevant in targeting the malignant plasma cells of multiple myeloma, which typically lack BAFF-R and express TACI and BCMA, the two receptors that are capable of responding to APRIL as well as BAFF. This limitation does not preclude the efficacy of these therapeutics in multiple myeloma, and, in fact, the existence of a hypothetical BAFF-R$^+$ myeloma stem cell with a less mature B phenotype would make belimumab an excellent approach. However, the malignant plasma cells of Waldenström's macroglobulinemia are a more promising target, in that they may express BAFF-R both more often and to a greater degree than the APRIL-responsive receptors [86]. In addition to several clinical trials in lupus and Sjögren's syndrome, a phase II trial of belimumab in symptomatic Waldenström's macroglobulinemia is currently recruiting participants.

10.4.2.2 Receptor-Targeted Therapy

There are also therapeutics targeting the receptor side of this cytokine network in development: LR131, a radiolabeled recombinant BAFF; anti-BR3, a monoclonal antibody to BAFF-R; and rGel/BLyS, a fusion protein combining a toxic agent with the binding domain of BAFF [87–89]. While the antibody targeting BAFF-R is unlikely to be useful in the only occasionally BAFF-R-expressing multiple myeloma plasma cells, the other two drugs have great potential to reach the malignant cells due to the consistent presence of at least one BBR. Of these, LR131 is the only one to have reached clinical trials, undergoing testing in non-Hodgkins lymphoma and multiple myeloma [90–92]. A major weakness of an approach targeting any BBR is the lack of specificity to the malignant cells. Normal B lineage cells express BBRs and typically to greater degrees than normal and malignant plasma cells and thus may suffer from unintended depletion as well as be more susceptible to the drugs than their malignant counterparts. Once the surface expression of the target receptor is established, some of these therapeutics are still limited by the activity of the receptors on these cells; BAFF-R's role in the survival of immature B cells is clear, well-established, and easily demonstrable, while its role in the survival of mature, peripheral B cells is much harder to detect.

10.4.2.3 Signaling-Targeted Therapy

Besides therapeutic approaches to myeloma directly targeting BAFF/APRIL network ligands and receptors, it is also possible to exploit this cytokine network

by taking aim at the intracellular mechanisms responsible for receptor signaling. Therapeutics in this class primarily inhibit NF-κB signaling, which is central to BAFF- and APRIL-induced signaling events, and may also be responsible for a degree of positive feedback by upregulating BBRs, particularly BAFF-R.

In addition to its connection to the BAFF/APRIL network, targeting the NF-κB pathway in multiple myeloma is a logical strategy worthy of concerted and rapid pursuit due to its intimate connection to immune cell signaling, survival, and gene expression. The normal, physiological, NF-κB-mediated immune pathways that initiate cell survival, proliferation, and activation are typically dependent on microenvironmental stimuli, including growth factors from neighboring cells and direct interactions with cell surface bound molecules. Recent studies have shown that in a subset of multiple myeloma patients, and even its precursor condition, monoclonal gammopathy of undetermined significance (MGUS), these pathways become constitutively active through a number of diverse mechanisms including mutations in TACI and TRAFs [93, 94].

Clinical NF-κB inhibition is a complex and vast area of ongoing research in its own right, employing numerous groundbreaking approaches reviewed recently [95–98] and including the proteasome inhibitors, which are postulated to work in large part through NF-κB inhibition. Despite some successes, NF-κB inhibition is not a curative agent in multiple myeloma and still lacks any efficacy in some patients. Identifying those cases with NF-κB mutations may allow more patient-specific chemotherapeutic regimens with fewer misdirected and ineffective treatments.

10.4.3 Additional Challenges Facing Therapeutic Intervention in the BAFF/APRIL Network in Multiple Myeloma

In addition to the specific hurdles described above, enthusiasm for therapeutics targeting the BAFF/APRIL network in multiple myeloma must be tempered by recognition of the differences between the B lineage cells of autoimmunity and those of malignancy. In autoimmune disorders, the B cells participating in, if not driving, the ongoing pathology are presumed to be fundamentally normal, in that they behave in ways consistent with a known understanding of B cell physiology. The clonal cells of malignancy are distinct in that they have become independent of the endogenous mechanisms of homeostatic control and behave in ways often completely divorced from their environment and the immune milieu. Thus, while targeting pathways crucial for the survival and proliferation of normal B cells is a logical approach in any B lineage cell-driven disease, the likelihood of the approach's success is much greater in autoimmunity than in malignancy.

10.5 Conclusion

The somewhat subtle quality of the approaches directly targeting BAFF/APRIL network members, effecting immune modulation rather than outright destruction of the target cells, is a liability in a traditional chemotherapeutic. However, the same quality may ultimately open new treatment avenues by gaining in precision what it lacks in lethality, for instance preventing the progression of premalignant conditions to outright malignancy, as in the transition from MGUS to MM. In MGUS and other premalignant conditions, the aberrant cells maintain a stable population under an unknown modified homeostatic mechanism, and thus may still be susceptible to some mechanisms of homeostatic control and manipulation, particularly of the type this class of therapeutics provides.

The incurable, terminal nature of multiple myeloma requires that we explore every therapeutic option to its full extent. The BAFF/APRIL cytokine network provides several therapeutic approaches in the ligands, receptors, and signaling molecules that mediate the cytokines' effects in both normal and malignant myeloma plasma cells. The ligand- and receptor-directed therapeutics undergoing clinical trials in this field are primarily driven by their application in autoimmunity, in which the cytokines and their interaction with their receptors are theorized to play an essential role in driving the disease. The application of therapeutic strategies across disease entities may indeed provide significant benefits in treating all malignancies of B lineage origin that share the quality of expressing one or more BBR, from the malignant cells of B cell acute lymphocytic leukemia to those of multiple myeloma. Our understanding of the role of this network in multiple myeloma suggests that the BBRs that characterize the malignant cells serve to augment the disease but not to control it, and thus these therapeutic approaches are likely to serve as adjuncts to a complex chemotherapeutic regimen rather than as a single curative agent. The agents targeting NF-κB, however, have the benefit of acting on a key mediator of diverse cellular processes advantageous to malignant cells regardless of NF-κB's current dependence on or independence of microenvironmental stimuli. In this regard, NF-κB is the most promising target in the BAFF/APRIL network in multiple myeloma.

Acknowledgments This work was supported by National Institutes of Health Grants CA105258 and CA062242 (to D.F.J.). We would also like to thank the numerous investigators whose work informed the review, but whose work was not specifically cited due to space constraints.

References

1. Moore PA, Belvedere O, Orr A et al (1999) BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. Science 285:260–263
2. Mukhopadhyay A, Ni J, Zhai Y et al (1999) Identification and characterization of a novel cytokine, THANK, a TNF homologue that activates apoptosis, nuclear factor-kappaB, and c-Jun NH2-terminal kinase. J Biol Chem 274:15978–15981

3. Schneider P, MacKay F, Steiner V et al (1999) BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. J Exp Med 189:1747–1756
4. Shu HB, Hu WH, Johnson H (1999) TALL-1 is a novel member of the TNF family that is down-regulated by mitogens. J Leukoc Biol 65:680–683
5. Tribouley C, Wallroth M, Chan V et al (1999) Characterization of a new member of the TNF family expressed on antigen presenting cells. Biol Chem 380:1443–1447
6. Gross JA, Johnston J, Mudri S et al (2000) TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease. Nature 404:995–999
7. Hahne M, Kataoka T, Schroter M et al (1998) APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth. J Exp Med 188:1185–1190
8. Kelly K, Manos E, Jensen G et al (2000) APRIL/TRDL-1, a tumor necrosis factor-like ligand, stimulates cell death. Cancer Res 60:1021–1027
9. Bossen C, Schneider P (2006) BAFF, APRIL and their receptors: structure, function and signaling. Semin Immunol 18:263–275
10. Gavin AL, Ait-Azzouzene D, Ware CF et al (2003) DeltaBAFF, an alternate splice isoform that regulates receptor binding and biopresentation of the B cell survival cytokine, BAFF. J Biol Chem 278:38220–38228
11. Pradet-Balade B, Medema JP, Lopez-Fraga M et al (2002) An endogenous hybrid mRNA encodes TWE-PRIL, a functional cell surface TWEAK-APRIL fusion protein. EMBO J 21:5711–5720
12. Gavin AL, Duong B, Skog P et al (2005) deltaBAFF, a splice isoform of BAFF, opposes full-length BAFF activity in vivo in transgenic mouse models. J Immunol 175:319–328
13. Kanakaraj P, Migone TS, Nardelli B et al (2001) BLyS binds to B cells with high affinity and induces activation of the transcription factors NF-kappaB and ELF-1. Cytokine 13:25–31
14. Karpusas M, Cachero TG, Qian F et al (2002) Crystal structure of extracellular human BAFF, a TNF family member that stimulates B lymphocytes. J Mol Biol 315:1145–1154
15. Lopez-Fraga M, Fernandez R, Albar JP et al (2001) Biologically active APRIL is secreted following intracellular processing in the Golgi apparatus by furin convertase. EMBO Rep 2:945–951
16. Wallweber HJA, Compaan DM, Starovasnik MA et al (2004) The crystal structure of a proliferation-inducing ligand, APRIL. J Mol Biol 343:283–290
17. Schwaller J, Went P, Matthes T et al (2007) Paracrine promotion of tumor development by the TNF ligand APRIL in Hodgkin's Disease. Leukemia 21:1324–1327
18. Huard B, McKee T, Bosshard C et al (2008) APRIL secreted by neutrophils binds to heparan sulfate proteoglycans to create plasma cell niches in human mucosa. J Clin Invest 118:2887–2895
19. Liu Y, Xu L, Opalka N et al (2002) Crystal structure of sTALL-1 reveals a virus-like assembly of TNF family ligands. Cell 108:383–394
20. Liu Y, Hong X, Kappler J et al (2003) Ligand-receptor binding revealed by the TNF family member TALL-1. Nature 423:49–56
21. Cachero TG, Schwartz IM, Qian F et al (2006) Formation of virus-like clusters is an intrinsic property of the tumor necrosis factor family member BAFF (B cell activating factor). Biochemistry 45:2006–2013
22. Pelletier M, Thompson JS, Qian F et al (2003) Comparison of soluble decoy IgG fusion proteins of BAFF-R and BCMA as antagonists for BAFF. J Biol Chem 278:33127–33133
23. Zhukovsky EA, Lee J-O, Villegas M et al (2004) TNF ligands: is TALL-1 a trimer or a virus-like cluster? Nature 427:413–414 (discussion 414)
24. Bossen C, Cachero TG, Tardivel A et al (2008) TACI, unlike BAFF-R, is solely activated by oligomeric BAFF and APRIL to support survival of activated B cells and plasmablasts. Blood 111:1004–1012
25. Ingold K, Zumsteg A, Tardivel A et al (2005) Identification of proteoglycans as the APRIL-specific binding partners. J Exp Med 201:1375–1383
26. Moreaux J, Sprynski A-C, Dillon SR et al (2009) APRIL and TACI interact with syndecan-1 on the surface of multiple myeloma cells to form an essential survival loop. Eur J Haematol 83:119–129

27. Nardelli B, Belvedere O, Roschke V et al (2001) Synthesis and release of B-lymphocyte stimulator from myeloid cells. Blood 97:198–204
28. Litinskiy MB, Nardelli B, Hilbert DM et al (2002) DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. Nat Immunol 3:822–829
29. Scapini P, Nardelli B, Nadali G et al (2003) G-CSF-stimulated neutrophils are a prominent source of functional BLyS. J Exp Med 197:297–302
30. Huard B, Arlettaz L, Ambrose C et al (2004) BAFF production by antigen-presenting cells provides T cell co-stimulation. Int Immunol 16:467–475
31. Gorelik L, Gilbride K, Dobles M et al (2003) Normal B cell homeostasis requires B cell activation factor production by radiation-resistant cells. J Exp Med 198:937–945
32. Novak AJ, Bram RJ, Kay NE et al (2002) Aberrant expression of B-lymphocyte stimulator by B chronic lymphocytic leukemia cells: a mechanism for survival. Blood 100:2973–2979
33. He B, Chadburn A, Jou E et al (2004) Lymphoma B cells evade apoptosis through the TNF family members BAFF/BLyS and APRIL. J Immunol 172:3268–3279 [Erratum appears in J Immunol. 2004 Apr. 15; 172(8): following 5127]
34. Kern C, Cornuel J-F, Billard C et al (2004) Involvement of BAFF and APRIL in the resistance to apoptosis of B-CLL through an autocrine pathway. Blood 103:679–688
35. Novak AJ, Darce JR, Arendt BK et al (2004) Expression of BCMA, TACI, and BAFF-R in multiple myeloma: a mechanism for growth and survival. Blood 103:689–694
36. Chu VT, Enghard P, Riemekasten G et al (2007) In vitro and in vivo activation induces BAFF and APRIL expression in B cells. J Immunol 179:5947–5957
37. Thompson JS, Bixler SA, Qian F et al (2001) BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. Science 293:2108–2111
38. Yan M, Brady JR, Chan B et al (2001) Identification of a novel receptor for B lymphocyte stimulator that is mutated in a mouse strain with severe B cell deficiency. Curr Biol 11:1547–1552
39. von Bulow GU, Bram RJ (1997) NF-AT activation induced by a CAML-interacting member of the tumor necrosis factor receptor superfamily. Science 278:138–141
40. Wu Y, Bressette D, Carrell JA et al (2000) Tumor necrosis factor (TNF) receptor superfamily member TACI is a high affinity receptor for TNF family members APRIL and BLyS. J Biol Chem 275:35478–35485
41. Xia XZ, Treanor J, Senaldi G et al (2000) TACI is a TRAF-interacting receptor for TALL-1, a tumor necrosis factor family member involved in B cell regulation. J Exp Med 192:137–143
42. Yan M, Marsters SA, Grewal IS et al (2000) Identification of a receptor for BLyS demonstrates a crucial role in humoral immunity. Nat Immunol 1:37–41
43. Yu G, Boone T, Delaney J et al (2000) APRIL and TALL-I and receptors BCMA and TACI: system for regulating humoral immunity. Nat Immunol 1:252–256
44. Laabi Y, Gras MP, Carbonnel F et al (1992) A new gene, BCM, on chromosome 16 is fused to the interleukin 2 gene by a t(4;16)(q26;p13) translocation in a malignant T cell lymphoma. EMBO J 11:3897–3904
45. Shu HB, Johnson H (2000) B cell maturation protein is a receptor for the tumor necrosis factor family member TALL-1. Proc Natl Acad Sci USA 97:9156–9161
46. Thompson JS, Schneider P, Kalled SL et al (2000) BAFF binds to the tumor necrosis factor receptor-like molecule B cell maturation antigen and is important for maintaining the peripheral B cell population. J Exp Med 192:129–135
47. Hatzoglou A, Roussel J, Bourgeade MF et al (2000) TNF receptor family member BCMA (B cell maturation) associates with TNF receptor-associated factor (TRAF) 1, TRAF2, and TRAF3 and activates NF-kappa B, elk-1, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase. J Immunol 165:1322–1330
48. Xu L-G, Shu H-B (2002) TNFR-associated factor-3 is associated with BAFF-R and negatively regulates BAFF-R-mediated NF-kappa B activation and IL-10 production. J Immunol 169:6883–6889
49. Ni C-Z, Oganesyan G, Welsh K et al (2004) Key molecular contacts promote recognition of the BAFF receptor by TNF receptor-associated factor 3: implications for intracellular signaling regulation. J Immunol 173:7394–7400

50. Hendriks J, Planelles L, de Jong-Odding J et al (2005) Heparan sulfate proteoglycan binding promotes APRIL-induced tumor cell proliferation. Cell Death Differ 12:637–648
51. Kimberley FC, van Bostelen L, Cameron K et al (2009) The proteoglycan (heparan sulfate proteoglycan) binding domain of APRIL serves as a platform for ligand multimerization and cross-linking. FASEB J 23:1584–1595
52. Sakurai D, Hase H, Kanno Y et al (2007) TACI regulates IgA production by APRIL in collaboration with HSPG. Blood 109:2961–2967
53. Ng LG, Sutherland APR, Newton R et al (2004) B cell-activating factor belonging to the TNF family (BAFF)-R is the principal BAFF receptor facilitating BAFF costimulation of circulating T and B cells. J Immunol 173:807–817
54. Darce JR, Arendt BK, Wu X et al (2007) Regulated expression of BAFF-binding receptors during human B cell differentiation. J Immunol 179:7276–7286
55. Groom JR, Fletcher CA, Walters SN et al (2007) BAFF and MyD88 signals promote a lupuslike disease independent of T cells. J Exp Med 204:1959–1971
56. Mihalcik SA, Huddleston PM 3rd, Wu X et al (2010) The Structure of the TNFRSF13C promoter enables differential expression of BAFF-R during B cell ontogeny and terminal differentiation. J Immunol 185(2):1045–1054
57. O'Connor BP, Raman VS, Erickson LD et al (2004) BCMA is essential for the survival of long-lived bone marrow plasma cells. J Exp Med 199:91–98
58. Avery DT, Kalled SL, Ellyard JI et al (2003) BAFF selectively enhances the survival of plasmablasts generated from human memory B cells. J Clin Invest 112:286–297 [Erratum appears in J Clin Invest. 113(7):1069]
59. Benson MJ, Dillon SR, Castigli E et al (2008) Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. J Immunol 180:3655–3659
60. Moreaux J, Legouffe E, Jourdan E et al (2004) BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone. Blood 103:3148–3157
61. Broyl A, Hose D, Lokhorst H et al (2010) Gene expression profiling for molecular classification of multiple myeloma in newly diagnosed patients. Blood. doi:10.1182/blood-2009-12-261032
62. Moreaux J, Cremer FW, Reme T et al (2005) The level of TACI gene expression in myeloma cells is associated with a signature of microenvironment dependence versus a plasmablastic signature. Blood 106:1021–1030
63. Moreaux J, Hose D, Jourdan M et al (2007) TACI expression is associated with a mature bone marrow plasma cell signature and C-MAF overexpression in human myeloma cell lines. Haematologica 92:803–811
64. Abe M, Kido S, Hiasa M et al (2006) BAFF and APRIL as osteoclast-derived survival factors for myeloma cells: a rationale for TACI-Fc treatment in patients with multiple myeloma. Leukemia 20:1313–1315
65. Tai Y-T, Li X-F, Breitkreutz I et al (2006) Role of B-cell-activating factor in adhesion and growth of human multiple myeloma cells in the bone marrow microenvironment. Cancer Res 66:6675–6682
66. Yaccoby S, Pennisi A, Li X et al (2008) Atacicept (TACI-Ig) inhibits growth of TACI(high) primary myeloma cells in SCID-hu mice and in coculture with osteoclasts. Leukemia 22:406–413
67. Batten M, Groom J, Cachero TG et al (2000) BAFF mediates survival of peripheral immature B lymphocytes. J Exp Med 192:1453–1466
68. Groom J, Kalled SL, Cutler AH et al (2002) Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome. J Clin Invest 109:59–68
69. Planelles L, Carvalho-Pinto CE, Hardenberg G et al (2004) APRIL promotes B-1 cell-associated neoplasm. Cancer Cell 6:399–408
70. Briones J, Timmerman JM, Hilbert DM et al (2002) BLyS and BLyS receptor expression in non-Hodgkin's lymphoma. Exp Hematol 30:135–141
71. Novak AJ, Grote DM, Stenson M et al (2004) Expression of BLyS and its receptors in B-cell non-Hodgkin lymphoma: correlation with disease activity and patient outcome. Blood 104: 2247–2253

72. Haiat S, Billard C, Quiney C et al (2006) Role of BAFF and APRIL in human B-cell chronic lymphocytic leukaemia. Immunology 118:281–292
73. Planelles L, Castillo-Gutierrez S, Medema JP et al (2007) APRIL but not BLyS serum levels are increased in chronic lymphocytic leukemia: prognostic relevance of APRIL for survival. Haematologica 92:1284–1285
74. Bojarska-Junak A, Hus I, Chocholska S et al (2009) BAFF and APRIL expression in B-cell chronic lymphocytic leukemia: correlation with biological and clinical features. Leuk Res 33:1319–1327
75. Molica S, Digiesi G, Battagliai C et al (2010) Baff Serum Level Predicts Time to First Treatment in Early Chronic Lymphocytic Leukemia. Eur J Haematol. doi:10.1111/j.1600-0609.2010.01482.x
76. Ryan MC, Grewal IS (2009) Targeting of BAFF and APRIL for autoimmunity and oncology. Adv Exp Med Biol 647:52–63
77. Rossi J-F, Moreaux J, Rose M et al (2006) A Phase I/II Study of Atacicept (TACI-Ig) To Neutralize APRIL and BLyS in Patients with Refractory or Relapsed Multiple Myeloma (MM) or Active Previously Treated Waldenstrom's Macroglobulinemia (WM). ASH Annual Meeting Abstracts 108:3578
78. Rossi JF, Moreaux J, Hose D et al (2009) Atacicept in relapsed/refractory multiple myeloma or active Waldenstrom's macroglobulinemia: a phase I study. Br J Cancer 101:1051–1058
79. Blade J, Samson D, Reece D et al (1998) Criteria for evaluating disease response and progression in patients with multiple myeloma treated by high-dose therapy and haemopoietic stem cell transplantation. Myeloma Subcommittee of the EBMT. European Group for Blood and Marrow Transplant. Br J Haematol 102:1115–1123
80. Dall'Era M, Chakravarty E, Wallace D et al (2007) Reduced B lymphocyte and immunoglobulin levels after atacicept treatment in patients with systemic lupus erythematosus: results of a multicenter, phase Ib, double-blind, placebo-controlled, dose-escalating trial. Arthritis Rheum 56:4142–4150
81. Munafo A, Priestley A, Nestorov I et al (2007) Safety, pharmacokinetics and pharmacodynamics of atacicept in healthy volunteers. Eur J Clin Pharmacol 63:647–656
82. Ansell SM, Witzig TE, Inwards DJ et al (2008) Phase I clinical study of atacicept in patients with relapsed and refractory B-cell non-Hodgkin's lymphoma. Clin Cancer Res 14:1105–1110
83. Nestorov I, Munafo A, Papasouliotis O et al (2008) Pharmacokinetics and biological activity of atacicept in patients with rheumatoid arthritis. J Clin Pharmacol 48:406–417
84. Tak PP, Thurlings RM, Rossier C et al (2008) Atacicept in patients with rheumatoid arthritis: results of a multicenter, phase Ib, double-blind, placebo-controlled, dose-escalating, single- and repeated-dose study. Arthritis Rheum 58:61–72
85. Pena-Rossi C, Nasonov E, Stanislav M et al (2009) An exploratory dose-escalating study investigating the safety, tolerability, pharmacokinetics and pharmacodynamics of intravenous atacicept in patients with systemic lupus erythematosus. Lupus 18:547–555
86. Elsawa SF, Novak AJ, Grote DM et al (2006) B-lymphocyte stimulator (BLyS) stimulates immunoglobulin production and malignant B-cell growth in Waldenstrom macroglobulinemia. Blood 107:2882–2888
87. Lyu M-A, Cheung LH, Hittelman WN et al (2007) The rGel/BLyS fusion toxin specifically targets malignant B cells expressing the BLyS receptors BAFF-R, TACI, and BCMA. Mol Cancer Ther 6:460–470
88. Nimmanapalli R, Lyu M-A, Du M et al (2007) The growth factor fusion construct containing B-lymphocyte stimulator (BLyS) and the toxin rGel induces apoptosis specifically in BAFF-R-positive CLL cells. Blood 109:2557–2564
89. Lyu MA, Rai D, Ahn KS et al (2010) The rGel/BLyS fusion toxin inhibits diffuse large B-cell lymphoma growth in vitro and in vivo. Neoplasia 12:366–375
90. Belch A, McEwan A, Hewitt J, et al. (2003) Early clinical data for LymphoRad-131 (LR131; Iodine I-131 labeled B-lymphocyte stimulator) in patients with relapsed/refractory non-Hodgkin's lymphoma. ASH Annual Meeting Abstracts: 1481.

91. Sung C, Stabin M, Brill AB, et al. (2003) LymphoRad-131 Pharmacokinetics and dosimetry in ongoing phase I multiple myeloma and non-Hodgkin's lymphoma trials. ASH Annual Meeting Abstracts: 2537.
92. Belch A, McEwan A, Hewitt J et al (2004) Tumor targeting, dosimetry and clinical response data for Lymphorad-131 (LR131; Iodine I-131 Labeled B-Lymphocyte Stimulator) in patients with relapsed/refractory non-Hodgkin's lymphoma. ASH Annual Meeting Abstracts 104:750
93. Annunziata CM, Davis RE, Demchenko Y et al (2007) Frequent engagement of the classical and alternative NF-kappaB pathways by diverse genetic abnormalities in multiple myeloma. Cancer Cell 12:115–130
94. Keats JJ, Fonseca R, Chesi M et al (2007) Promiscuous mutations activate the noncanonical NF-kappaB pathway in multiple myeloma. Cancer Cell 12:131–144
95. Lee CH, Jeon Y-T, Kim S-H et al (2007) NF-kappaB as a potential molecular target for cancer therapy. Biofactors 29:19–35
96. Baud V, Karin M (2009) Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. Nat Rev Drug Discov 8:33–40
97. Tas SW, Vervoordeldonk MJBM, Tak PP (2009) Gene therapy targeting nuclear factor-kappaB: towards clinical application in inflammatory diseases and cancer. Curr Gene Ther 9:160–170
98. Lin Y, Bai L, Chen W et al (2010) The NF-kappaB activation pathways, emerging molecular targets for cancer prevention and therapy. Expert Opin Ther Targets 14:45–55

Chapter 11
Role of Osteoblast in Myeloma Pathology

Sonia Vallet and Noopur Raje

Abstract Bone disease in multiple myeloma results from uncoupled osteoclast and osteoblast activity. Osteoblasts or bone forming cells show impaired differentiation, increased apoptosis and overall reduced function. Soluble and membrane bound proteins have been studied for their role in myeloma-related osteoblast inhibition. Progress in the understanding of the pathogenesis of the osteoblast axis in myeloma has allowed the development of novel targeted therapies, such as DKK1 and activin inhibitors. This chapter provides an overview of the mechanisms behind osteoblast inhibition in myeloma and novel anabolic agents under clinical investigation.

11.1 Introduction

Multiple myeloma (MM) is a hematological cancer characterized by malignant plasma cells homing and proliferating in the bone marrow microenvironment. The estimated incidence of MM in the USA in 2010 was nearly 20,000 new cases. Although prolonged overall survival has been achieved with new therapeutic agents, there is still no evidence of cure and more than 10,000 patients die each year in the USA from MM-related complications [1]. The occurrence of complications such as pathological fractures consequent to bone disease contribute to this mortality rate. About 80% of MM patients develop osteolytic lesions, which can also present with severe pain and vertebral body compression fractures [2, 3].

The pathogenesis of bone disease relies on the interplay between tumor cells and the bone microenvironment. Bone marrow (BM) biopsies from MM patients revealed a generalized osteoclast (OC) activation correlating with tumor burden [4, 5], and a

S. Vallet, M.D. • N. Raje, M.D. (✉)
Division of Hematology-Oncology, Massachusetts General Hospital Cancer Center, Harvard Medical School, Massachusetts General Hospital, 55 Fruit Street, 02114 Boston MA, USA
e-mail: nraje@partners.org

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_11,

decrease in trabecular formation and calcification rate, suggesting impaired osteoblast (OB) activity [6]. Indeed, myeloma cells disrupt bone homeostasis by altering the balance between bone-resorbing OC and bone-forming OB. They stimulate osteoclastogenesis and inhibit OB differentiation. In addition, tumor cells stimulate angiogenesis, and alter the cytokine profile in the bone marrow (BM), favoring the release of angiogenic, chemotactic, and growth cytokines as well as OC-activating factors (OAFs) [7–9]. In turn, OCs together with bone marrow stromal cells (BMSC) and endothelial cells support plasma cell proliferation and mediate chemoresistance [10]. Conversely, OBs have an overall inhibitory effect on tumor cell proliferation [11, 12]. These changes in cell composition and function within the BM milieu induced by malignant plasma cells create a niche permissive to cancer propagation.

Treatment strategies currently available for MM patients with bone disease are largely palliative as they aim at alleviating pain and reducing the incidence of skeletal-related issues such as vertebral fractures. They include radiation, surgical fixation, and bisphosphonate administration. In order to treat bone disease effectively, there is a need for therapies with the ability to reverse the osteolytic process and restore physiologic bone remodeling. Ongoing studies therefore attempt to unravel the pathogenesis of bone lesions in MM with the goal of identifying novel therapeutically relevant targets.

11.2 Physiology of Osteoblast

OBs play a pivotal role in the maintenance of bone health. They derive from common mesenchymal progenitor cells along with adipocytes, chondrocytes, and myocytes. Three principal periods of differentiation can be identified in vitro (1) nonfunctional preosteoblasts; (2) OB secreting extracellular matrix; and (3) OBs inducing bone mineralization. In vivo three different functional states can be recognized: active bone-forming cells, quiescent bone-lining cells, and regulatory bone-embedded osteocytes [13]. Quiescent and active OBs lie on the bone surface and together with OCs regulate the bone remodeling process. In the absence of bone formation, inactive OBs are recognizable as lining cells. Bone-resorbing OCs activate OBs, identifiable as osteoid-secreting cubic cells. During bone formation, some OBs remain trapped in growing bone, forming the osteocytes that function as mechanical receptors directing the process of bone remodeling according to stress forces [14].

Regulators of osteogenesis include systemic and locally secreted factors, like parathyroid hormone (PTH), fibroblast growth factor (FGF), Wnt, and BMP (bone morphogenetic proteins) [15]. Importantly, OB differentiation and function are coupled to osteoclastogenesis by means of surface and secreted bidirectional signaling, such as the Ephrin/Eph pathway. Stimulation of the receptor EphB4 on OB surface via the membrane-bound ligand ephrinB2 expressed by OCs promotes osteoblastogenesis. In turn, reverse signaling via ephrinB2 inhibits OC differentiation [16], thus regulating bone remodeling. Other important OB-derived modulators of OC activity are RANKL and its antagonist osteoprotegerin (OPG), which respectively induce and inhibit OC differentiation.

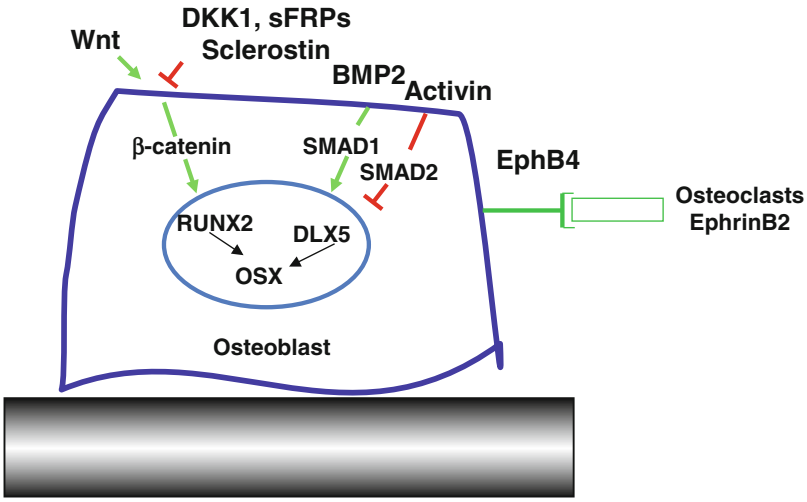


Fig. 11.1 Therapeutically amenable pathways directing OB differentiation. Osteogenesis is regulated by several signaling pathways that may exert opposite effects on critical transcription factors. Two main signaling pathways promote OB differentiation: the β-catenin and SMAD1 pathways that are downstream of Wnt and BMP, respectively. They modulate RUNX2, Osterix, and DLX5 transcriptional activity involved in progenitor cell commitment to the OB lineage and cell maturation. Inhibitors of the Wnt pathway are DKK1, sclerostin, and soluble frizzled-related proteins. The balanced activity of SMAD1 and SMAD2, induced by BMPs and activin, regulates DLX5 expression. Other recently identified modulators of OB differentiation are Ephrin ligands and receptors that contribute to the OB/OC coupling by simultaneously stimulating osteogenesis and inhibiting OCs

In vitro osteogenesis studies have allowed the identification of the signaling pathways and transcription factors regulating OB differentiation (Fig. 11.1).

11.2.1 Wnt Signaling

Via modulation of the receptors such as LRP5/6 and frizzled, Wnt and its antagonists, dickopff (DKK)1, sclerostin and soluble frizzled-related protein (sFPR)-1,2,3,4 regulate the canonical β-catenin-dependent, and non-canonical disheveled or calcium-dependent pathways [17]. Ultimately, these signaling pathways converge to upregulate runt-related transcription factor (Runx)2 and osterix transcription factor activity [18, 19]. Runx2 is critical to progenitor cell commitment to the OB lineage and modulates several bone matrix protein genes [20]. Arrest in OB differentiation and lack of bone formation have been demonstrated in RUNX2 knockout mice [21]. Importantly, overexpression of RUNX2 induces osteopenia as well because OBs are frozen in an immature state, suggesting that timely RUNX2 activation is critical in osteogenesis [22]. Osterix is downstream of RUNX2 and contributes to early OB differentiation. In fact, osterix null mice have endochondral skeleton in the absence

of bone formation [23]. Osterix is also critical to OB function, since postnatal gene knockout reduces bone formation rate and mineralization without affecting OB proliferation or differentiation [24].

11.2.2 BMP Signaling

BMPs belong to the TGFβ superfamily; they activate dimeric receptors (activin and BMP receptors) and downstream signaling pathways that exert opposite effects on bone formation. BMP2 stimulates OB differentiation via SMAD1 and distal-less homeobox (DLX)5 upregulation [25]. In contrast, activin is a negative regulator as it inhibits DLX5 by means of SMAD2 activation. DLX5 is a critical transcription factor in OB differentiation. It contributes to the regulation of osterix expression [26] and lack of DLX5 gene expression induces abnormal osteogenesis. *DLX5* is also a common target of the β-catenin signaling pathway [27, 28], so that differential effects on DLX5 transcription account for the opposing effects of Wnt10b, BMP2, activin, and TGF-β on OB differentiation [29, 30].

11.3 Osteoblast–Myeloma Interactions

The development of osteolytic lesions in MM relies on impaired OB function that cannot compensate for the OC hyperactivity. Indeed, tumor cells interfere with OB differentiation and activity by means of cell contact and cytokine secretion. The VLA4/VCAM-1 interaction downregulates RUNX2 activity in OB progenitor cells [31], and inhibition of this signaling via neutralizing antibodies promotes OB differentiation. In addition, several soluble factors have been identified as OB inhibitors, such as DKK1 and frizzle-related protein (sFRP) 2 and 3, activin and TGF-β, CCL3, interleukins (IL)-3 and 7. Along with these mechanisms, a deregulation of the ephrin signaling pathway has also been demonstrated in MM.

11.3.1 DKK1 and sFRPs

The WNT signaling antagonist DKK1 is upregulated in BM plasma of MM patients with osteolytic lesions [32]. Secreted by MM cells, DKK1 downregulates RUNX2 activity most likely via the non-canonical Wnt signaling pathway since its levels are independent from β-catenin activation status [33]. Both in vitro and in vivo studies show that DKK1 inhibition promotes OB differentiation and function [34, 35]. In addition to DKK1, sFRP2 and 3 are expressed by MM cells and upregulated in BM plasma of MM patients with bone lesions and may contribute to OB inhibition [33, 36].

11.3.2 Activin A

Like DKK1, activin A is also upregulated in MM patients with osteolytic lesions. Tumor cells do not directly produce activin but stimulate its secretion by BMSC. Activin upregulates SMAD2 signaling, resulting in inhibition of DLX5 with subsequent impairment of osteoblastogenesis. The in vivo relevance of this pathway is demonstrated by the correlation between DLX5 downregulation in BM biopsies of MM patients with bone disease and high BM plasma activin A levels [30]. In addition, activin exerts a pro-OC effect, and its inhibition results in overall improvement of bone disease in several cancer mouse models [30, 37].

11.3.3 TGFβ and Interleukins

Similar to activin A, TGFβ inhibits differentiation of mesenchymal stem cells to osteoblasts as well as adipocytes, and its inhibition restores osteogenesis and suppresses MM cell growth [38]. IL-3 is secreted by both malignant plasma cells and T lymphocytes in MM. It stimulates OC differentiation in combination with RANKL. It is a growth factor for MM cells and indirectly inhibits OB differentiation via stimulation of CD45+ monocytic–macrophagic cell population [39–41]. IL-7 is also a MM-derived cytokine stimulating RANKL production by T lymphocytes [42] and mediating MM-induced OB inhibition via downregulation of RUNX2 transcriptional activity [31].

11.3.4 Ephrin Signaling

A deregulation of the EphrinB2/EphB4 signaling is recognizable in MM. BMSC from MM patients have lower expression levels of both ligand and receptor, and tumor cells are directly responsible for this effect. In vivo treatment with chimeric ephrinB2-Fc stimulated angiogenesis, osteoblastogenesis, and bone formation. In addition to these effects, treatment with EphB4 inhibits tumor growth, osteoclastogenesis, and angiogenesis [43].

In contrast to OCs and BMSC that support tumor cells growth, OBs inhibit directly and indirectly MM cell survival. In vitro coculture studies suggested MM growth inhibition in the presence of OB, and similarly upregulation of the β-catenin signaling pathway results in tumor growth inhibition in vivo [44]. Although the mechanism of MM inhibition has still to be clarified, small leucine-rich proteoglycans are probably involved. Decorin, in particular, is an OB-derived extracellular matrix component that induces MM cell apoptosis via p21 activation and inhibits angiogenesis and osteoclastogenesis [45]. In addition, OBs affect tumor cell growth indirectly via their regulatory effects on OCs. The OPG expression in OBs is inhibited

by MM-derived DKK1, whereas RANKL secretion is upregulated [46]. Therefore, stimulating OB differentiation may inhibit OC differentiation and subsequently their support to MM cells.

Of interest, mesenchymal cells and OBs derived from healthy donors or myeloma patients show differences in their cytokine and gene expression profile as well as function. Ex vivo cultures of MM-derived BMSC express factors favorable to tumor growth and the OBs generated from these cells are unable to differentiate in functional cells [47–49]. A recent study could not identify any primary karyotype or telomerase defects in MM-derived MSC, suggesting that these differences may be consequent to an imprinting induced by tumor cells in vivo [50].

11.4 Therapeutically Targeting Osteoblasts

Current therapies to arrest bone disease and prevent skeletal complications include bisphosphonates (BPs) and radiotherapy. However, these strategies do not restore normal bone architecture. Since recent advances in MM treatment have resulted in improved patient overall survival, targeting bone disease has taken on a new relevance as the focus is now largely on quality of life. Several studies suggest that manipulating the BM niche may result in balanced bone homeostasis and consequent tumor growth inhibition. Therefore, the identification of novel agents to overcome bone disease and, eventually, treat MM is an important clinical challenge (Fig. 11.2).

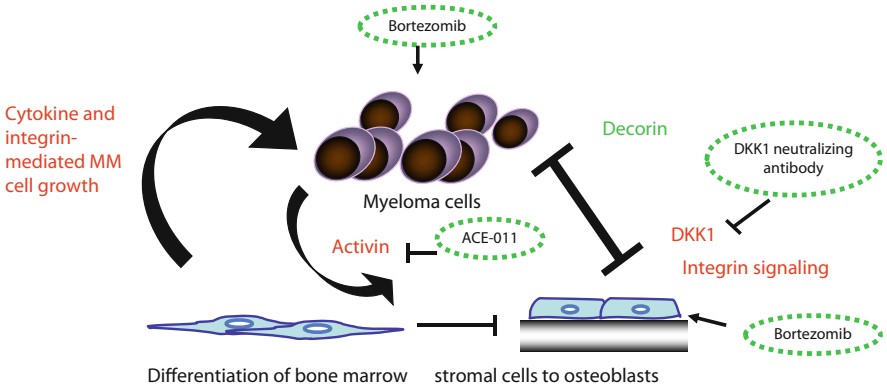


Fig. 11.2 *Anabolic agents*. Bortezomib is a potent anti-MM agent with anabolic effects due to RUNX2 upregulation. DKK1 neutralizing antibodies are anabolic agents currently undergoing clinical evaluation in MM in combination with Zoledronic acid. ACE-011 is a humanized soluble receptor inhibiting activin A with a promising dual activity as osteoblast stimulator and osteoclast inhibitor. Ongoing clinical trials are assessing its effects on bone architecture in MM

11.4.1 Sotatercept (ACE-011)

Activin A is a multifunctional cytokine involved in fetal development, gonadal and bone homeostasis, and erythropoiesis as well. High levels of this cytokine have been identified in MM, prostate and breast cancer patients. In MM, in particular, activin A expression correlates with osteolytic burden. In vitro inhibition of activin A using a murine chimeric antibody RAP-011 (Acceleron Pharma, Cambridge, MA) results in increased OB differentiation and decreased OC development, restoring the bone balance in MM. In animal models of osteoporosis, MM and breast cancer associated-bone disease RAP-011 treatment improved bone density, prevented osteolytic bone disease, and reduced tumor growth [30, 37, 51]. After a promising phase I trial in post-menopausal women, the bone anabolic effects of the humanized activin A inhibitor Sotatercept (ACE-011) have been assessed in MM patients with osteolytic lesions receiving standard chemotherapy (NCT00747123). In this phase II study, increase in bone formation parameters and reduction in pain have been observed [52]. Of note in 75% of the patients treated with high doses of Sotatercept, an increase in Hb level has also been noted. As a result, a second phase II trial in patients with metastatic breast cancer (NCT00931606) addressing the hematopoietic effects of ACE-011 has been initiated.

Bortezomib has a direct cytotoxic effect on MM cells via proteasome and NF-kB signaling pathway inhibition and also indirectly by inhibition of MM-BMSC interactions. Several studies have identified bone effects resulting from OC inhibition and enhancement of OB differentiation. The suggested mechanism for the anti-OC effects of bortezomib consists of early p38 inhibition followed by a late impairment of NF-kB signaling and AP1 [53]. In contrast, the anabolic properties rely on the inhibition of proteasome activity and resultant RUNX2 and osterix upregulation [54–56]. In MM patients, bortezomib treatment with or without dexamethasone induces upregulation of OB activation and downregulation of bone-resorption markers [57, 58]. Notably, combination with alkylating agents and thalidomide (VMTD) diminishes benefits of bortezomib on OB differentiation [59]. Therefore, continued assessment of the effects of anti-MM strategies on bone remodeling are needed and may help identify optimal combination strategies.

11.4.2 DKK1 Antagonists

DKK1, a negative regulator of OB differentiation, is overexpressed in MM patients [32], and strategies to block DKK1 activity have been investigated. BHQ880 is a neutralizing antibody against DKK1 that promotes OB differentiation by reversing the negative effect of MM cells on OB formation and inhibits IL6 production by BMSC, therefore blocking the proliferative advantage conferred to tumor cells [34]. In vivo studies using both murine and humanized models of MM-bone disease demonstrated bone-anabolic properties of DKK1 inhibition, with increased bone formation, increased OB number, and improvement of osteolytic lesions [35, 60].

Importantly, blocking DKK1 resulted in reduced tumor growth, mainly as an indirect effect via modification of the tumor microenvironment [34]. A clinical trial (NCT00741377) is currently ongoing in relapsed or refractory myeloma patients to assess safety and tolerability as well as efficacy of the DKK1 inhibitor BHQ880 in combination with standard chemotherapy with or without bisphosphonates.

11.4.3 Sclerostin Antagonists

Sclerostin antagonists are currently under investigation for their anabolic effects. Mutations of sclerostin receptor (LRP5) cause an increased bone mass syndrome, and gene mutations abolishing sclerostin expression are responsible for sclerostosis [61], characterized by increased bone mineral density. High sclerostin levels have been reported in MM patients correlating with advanced disease, suggesting its involvement in MM pathogenesis [62]. Anti-sclerostin antibodies have been therefore developed and a phase I trial in healthy men and postmenopausal women demonstrated increases in bone mineral density at the lumbar spine and total hip compared with placebo [63].

11.5 Future Perspective

In the past few years, our knowledge on the pathogenesis of bone lesions in MM has greatly improved. Based on recent findings on tumor and bone cell interactions, novel targeted therapies are currently under clinical investigation. Future research challenges include the identification of patient populations at risk for bone lesions and the assessment of effective mechanism-based drug combination to reverse bone lesions.

Although almost all MM patients present with bone disease, not all of them develop complications. Therefore, tailored treatment strategies based on prognostic factors may improve patient quality of life. Recently, it has been shown that a combined analysis of C-terminal telopeptide of collagen type I (marker of osteoclast activity) and bone alkaline phosphatase (marker of osteoblast activity) identify patients at high risk of skeletal disease progression that may benefit from aggressive therapies [64].

Since bone disease is the result of an imbalanced OC/OB axis, treatments should aim at targeting both components. For example, lenalidomide inhibits OCs and may synergize with anabolic agents. Similarly, bisphosphonates represent the backbone strategy against osteolytic lesions in MM for their anti-catabolic effect. However, reports on a possible negative impact on osteoblast differentiation provide the rationale for clinical evaluation in combination with anabolic drugs such as DKK1.

In conclusion, balanced OB activity is important not only to restore bone homeostasis but is also critical to tumor cell inhibition. Strategies to rescue OB function in MM will soon become a relevant part in the treatment of MM.

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ (2009) Cancer statistics, 2009. CA Cancer J Clin 59(4):225–249
2. Coleman RE (1997) Skeletal complications of malignancy. Cancer 80(8 Suppl):1588–1594
3. Saad F, Lipton A, Cook R, Chen YM, Smith M, Coleman R (2007) Pathologic fractures correlate with reduced survival in patients with malignant bone disease. Cancer 110(8):1860–1867
4. Valentin-Opran A, Charhon SA, Meunier PJ, Edouard CM, Arlot ME (1982) Quantitative histology of myeloma-induced bone changes. Br J Haematol 52(4):601–610
5. Taube T, Beneton MN, McCloskey EV, Rogers S, Greaves M, Kanis JA (1992) Abnormal bone remodelling in patients with myelomatosis and normal biochemical indices of bone resorption. Eur J Haematol 49(4):192–198
6. Bataille R, Chappard D, Marcelli C, Dessauw P, Sany J, Baldet P et al (1989) Mechanisms of bone destruction in multiple myeloma: the importance of an unbalanced process in determining the severity of lytic bone disease. J Clin Oncol 7(12):1909–1914
7. Lentzsch S, Gries M, Janz M, Bargou R, Dorken B, Mapara MY (2003) Macrophage inflammatory protein 1-alpha (MIP-1 alpha) triggers migration and signaling cascades mediating survival and proliferation in multiple myeloma (MM) cells. Blood 101(9):3568–3573
8. Podar K, Anderson KC (2005) The pathophysiologic role of VEGF in hematologic malignancies: therapeutic implications. Blood 105(4):1383–1395
9. Alsayed Y, Ngo H, Runnels J, Leleu X, Singha UK, Pitsillides CM et al (2007) Mechanisms of regulation of CXCR4/SDF-1 (CXCL12)-dependent migration and homing in multiple myeloma. Blood 109(7):2708–2717
10. Roodman GD (2006) New potential targets for treating myeloma bone disease. Clin Cancer Res 12(20 Pt 2):6270s–6273s
11. Yaccoby S, Wezeman MJ, Zangari M, Walker R, Cottler-Fox M, Gaddy D et al (2006) Inhibitory effects of osteoblasts and increased bone formation on myeloma in novel culture systems and a myelomatous mouse model. Haematologica 91(2):192–199
12. Giuliani N, Rizzoli V, Roodman GD (2006) Multiple myeloma bone disease: pathophysiology of osteoblast inhibition. Blood 108(13):3992–3996
13. Komori T (2006) Regulation of osteoblast differentiation by transcription factors. J Cell Biochem 99(5):1233–1239
14. Franz-Odendaal TA, Hall BK, Witten PE (2006) Buried alive: how osteoblasts become osteocytes. Dev Dyn 235(1):176–190
15. Sims NA, Gooi JH (2008) Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption. Semin Cell Dev Biol 19(5):444–451
16. Zhao C, Irie N, Takada Y, Shimoda K, Miyamoto T, Nishiwaki T et al (2006) Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis. Cell Metab 4(2):111–121
17. Giuliani N, Mangoni M, Rizzoli V (2009) Osteogenic differentiation of mesenchymal stem cells in multiple myeloma: identification of potential therapeutic targets. Exp Hematol 37(8):879–886
18. Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS et al (2005) Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. J Biol Chem 280(39):33132–33140
19. Takada I, Mihara M, Suzawa M, Ohtake F, Kobayashi S, Igarashi M et al (2007) A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. Nat Cell Biol 9(11):1273–1285
20. Komori T (2009) Regulation of bone development and extracellular matrix protein genes by RUNX2. Cell Tissue Res 339(1):189–195
21. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K et al (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89(5):755–764
22. Liu W, Toyosawa S, Furuichi T, Kanatani N, Yoshida C, Liu Y et al (2001) Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. J Cell Biol 155(1):157–166

23. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR et al (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108(1):17–29
24. Baek WY, de Crombrugghe B, Kim JE (2010) Postnatally induced inactivation of Osterix in osteoblasts results in the reduction of bone formation and maintenance. Bone 46(4):920–928
25. Ryoo HM, Lee MH, Kim YJ (2006) Critical molecular switches involved in BMP-2-induced osteogenic differentiation of mesenchymal cells. Gene 366(1):51–57
26. Samee N, Geoffroy V, Marty C, Schiltz C, Vieux-Rochas M, Levi G et al (2008) Dlx5, a positive regulator of osteoblastogenesis, is essential for osteoblast-osteoclast coupling. Am J Pathol 173(3):773–780
27. Holleville N, Quilhac A, Bontoux M, Monsoro-Burq AH (2003) BMP signals regulate Dlx5 during early avian skull development. Dev Biol 257(1):177–189
28. Bennett CN, Longo KA, Wright WS, Suva LJ, Lane TF, Hankenson KD et al (2005) Regulation of osteoblastogenesis and bone mass by Wnt10b. Proc Natl Acad Sci USA 102(9):3324–3329
29. Lee MH, Kim YJ, Kim HJ, Park HD, Kang AR, Kyung HM et al (2003) BMP-2-induced Runx2 expression is mediated by Dlx5, and TGF-beta 1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression. J Biol Chem 278(36):34387–34394
30. Vallet S, Mukherjee S, Vaghela N, Hideshima T, Fulciniti M, Pozzi S et al (2010) Activin A promotes multiple myeloma-induced osteolysis and is a promising target for myeloma bone disease. PNAS 107(11):5124–5129
31. Giuliani N, Colla S, Morandi F, Lazzaretti M, Sala R, Bonomini S et al (2005) Myeloma cells block RUNX2/CBFA1 activity in human bone marrow osteoblast progenitors and inhibit osteoblast formation and differentiation. Blood 106(7):2472–2483
32. Tian E, Zhan F, Walker R, Rasmussen E, Ma Y, Barlogie B et al (2003) The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. N Engl J Med 349(26):2483–2494
33. Giuliani N, Morandi F, Tagliaferri S, Lazzaretti M, Donofrio G, Bonomini S et al (2007) Production of Wnt inhibitors by myeloma cells: potential effects on canonical Wnt pathway in the bone microenvironment. Cancer Res 67(16):7665–7674
34. Fulciniti M, Tassone P, Hideshima T, Vallet S, Nanjappa P, Ettenberg SA et al (2009) Anti-DKK1 mAb (BHQ880) as a potential therapeutic agent for multiple myeloma. Blood 114(2): 371–379
35. Heath DJ, Chantry AD, Buckle CH, Coulton L, Shaughnessy JD, Evans HR et al (2008) Inhibiting Dickkopf-1 (Dkk1) removes suppression of bone formation and prevents the development of osteolytic bone disease in multiple myeloma. J Bone Miner Res 24(3):425–436
36. Oshima T, Abe M, Asano J, Hara T, Kitazoe K, Sekimoto E et al (2005) Myeloma cells suppress bone formation by secreting a soluble Wnt inhibitor, sFRP-2. Blood 106(9):3160–3165
37. Chantry AD, Heath D, Mulivor AW, Pearsall S, Baud'huin M, Coulton L et al (2010) Inhibiting activin-A signaling stimulates bone formation and prevents cancer induced bone destruction in vivo. J Bone Miner Res 25(12):2633–2646
38. Takeuchi K, Abe M, Hiasa M, Oda A, Amou H, Kido S et al (2010) Tgf-Beta inhibition restores terminal osteoblast differentiation to suppress myeloma growth. PLoS One 5(3):e9870
39. Lee JW, Chung HY, Ehrlich LA, Jelinek DF, Callander NS, Roodman GD et al (2004) IL-3 expression by myeloma cells increases both osteoclast formation and growth of myeloma cells. Blood 103(6):2308–2315
40. Ehrlich LA, Cung HY, Ghobrial I, Choi SJ, Morandi F, Colla S et al (2005) IL-3 is a potential inhibitor of osteoblast differentiation in multiple myeloma. Blood 106(4):1407–1414
41. Giuliani N, Morandi F, Tagliaferri S, Colla S, Bonomini S, Sammarelli G et al (2006) Interleukin-3 (IL-3) is overexpressed by T lymphocytes in multiple myeloma patients. Blood 107(2):841–842
42. Giuliani N, Colla S, Sala R, Moroni M, Lazzaretti M, La Monica S et al (2002) Human myeloma cells stimulate the receptor activator of nuclear factor-kappa B ligand (RANKL) in T lymphocytes: a potential role in multiple myeloma bone disease. Blood 100(13):4615–4621

43. Pennisi A, Ling W, Li X, Khan S, Shaughnessy JD Jr, Barlogie B et al (2009) The ephrinB2/EphB4 axis is dysregulated in osteoprogenitors from myeloma patients and its activation affects myeloma bone disease and tumor growth. Blood 114(9):1803–1812
44. Edwards CM, Edwards JR, Lwin ST, Esparza J, Oyajobi BO, McCluskey B et al (2008) Increasing Wnt signaling in the bone marrow microenvironment inhibits the development of myeloma bone disease and reduces tumor burden in bone in vivo. Blood 111(5):2833–2842
45. Li X, Pennisi A, Yaccoby S (2008 Jul 1) Role of decorin in the antimyeloma effects of osteoblasts. Blood 112(1):159–168
46. Qiang YW, Chen Y, Stephens O, Brown N, Chen B, Epstein J et al (2008) Myeloma-derived Dickkopf-1 disrupts Wnt-regulated osteoprotegerin and RANKL production by osteoblasts: a potential mechanism underlying osteolytic bone lesions in multiple myeloma. Blood 112(1): 196–207
47. Wallace SR, Oken MM, Lunetta KL, Panoskaltsis-Mortari A, Masellis AM (2001) Abnormalities of bone marrow mesenchymal cells in multiple myeloma patients. Cancer 91(7):1219–1230
48. Silvestris F, Cafforio P, Calvani N, Dammacco F (2004) Impaired osteoblastogenesis in myeloma bone disease: role of upregulated apoptosis by cytokines and malignant plasma cells. Br J Haematol 126(4):475–486
49. Corre J, Mahtouk K, Attal M, Gadelorge M, Huynh A, Fleury-Cappellesso S et al (2007) Bone marrow mesenchymal stem cells are abnormal in multiple myeloma. Leukemia 21(5):1079–1088
50. Giuliani N, Lisignoli G, Novara F, Storti P, Zaffaroni N, Villa R et al (2010) Bone osteoblastic and mesenchymal stromal cells lack primarily tumoral features in multiple myeloma patients. Leukemia 24(7):1368–1370
51. Pearsall RS, Canalis E, Cornwall-Brady M, Underwood KW, Haigis B, Ucran J et al (2008) A soluble activin type IIA receptor induces bone formation and improves skeletal integrity. Proc Natl Acad Sci USA 105(19):7082–7087
52. Abdulkadyrov KM, Salogub GN, Khuazheva NK, Woolf R, Haltom E, Borgstein NG, Knight R, Renshaw G, Yang Y, Sherman ML (2009) ACE-011, a soluble activin receptor type Iia IgG-Fc fusion protein, increases hemoglobin (Hb) and improves bone lesions in multiple myeloma patients receiving myelosuppressive chemotherapy: preliminary analysis. Blood 114:1
53. von Metzler I, Krebbel H, Hecht M, Manz RA, Fleissner C, Mieth M et al (2007) Bortezomib inhibits human osteoclastogenesis. Leukemia 21(9):2025–2034
54. Giuliani N, Morandi F, Tagliaferri S, Lazzaretti M, Bonomini S, Crugnola M et al (2007) The proteasome inhibitor bortezomib affects osteoblast differentiation in vitro and in vivo in multiple myeloma patients. Blood 110(1):334–338
55. Mukherjee S, Raje N, Schoonmaker JA, Liu JC, Hideshima T, Wein MN et al (2008) Pharmacologic targeting of a stem/progenitor population in vivo is associated with enhanced bone regeneration in mice. J Clin Invest 118(2):491–504
56. DeMatteo M, Brunetti AE, Maiorano E, Cafforio P, Dammacco F, Silvestris F (2010) Constitutive down-regulation of Osterix in osteoblasts from myeloma patients: in vitro effect of Bortezomib and Lenalidomide. Leuk Res 34(2):243–249
57. Zangari M, Esseltine D, Lee CK, Barlogie B, Elice F, Burns MJ et al (2005) Response to bortezomib is associated to osteoblastic activation in patients with multiple myeloma. Br J Haematol 131(1):71–73
58. Terpos E, Heath DJ, Rahemtulla A, Zervas K, Chantry A, Anagnostopoulos A et al (2006) Bortezomib reduces serum dickkopf-1 and receptor activator of nuclear factor-kappaB ligand concentrations and normalises indices of bone remodelling in patients with relapsed multiple myeloma. Br J Haematol 135(5):688–692
59. Terpos E, Kastritis E, Roussou M, Heath D, Christoulas D, Anagnostopoulos N et al (2008) The combination of bortezomib, melphalan, dexamethasone and intermittent thalidomide is an effective regimen for relapsed/refractory myeloma and is associated with improvement of abnormal bone metabolism and angiogenesis. Leukemia 22(12):2247–2256

60. Yaccoby S, Ling W, Zhan F, Walker R, Barlogie B, Shaughnessy JD Jr (2007) Antibody-based inhibition of DKK1 suppresses tumor-induced bone resorption and multiple myeloma growth in vivo. Blood 109(5):2106–2111
61. Balemans W, Ebeling M, Patel N, Van Hul E, Olson P, Dioszegi M et al (2001) Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). Hum Mol Genet 10(5):537–543
62. Terpos E, Katodritou E, Bratengeier C, Lindner B, Harmelin S, Hawa G, Boutsikas G, Migkou M, Gavriatopoulou M, Michalis E, Pouli A, Kastritis E, Zervas K, Dimopoulos MA (2009) High serum sclerostin correlates with advanced stage, increased bone resorption, reduced osteoblast function, and poor survival in newly-diagnosed patients with multiple myeloma. Blood 114:425
63. Padhi D, Jang G, Stouch B, Fang L, Posvar E (2011) Single-dose, placebo-controlled, randomized study of AMG 785, a sclerostin monoclonal antibody. J Bone Miner Res 26(1):19–26
64. Lund T, Abildgaard N, Andersen TL, Delaisse JM, Plesner T (2010) Multiple myeloma: changes in serum C-terminal telopeptide of collagen type I and bone-specific alkaline phosphatase can be used in daily practice to detect imminent osteolysis. Eur J Haematol 84(5):412–420

Chapter 12
Migration and Homing in Multiple Myeloma

Giada Bianchi and Irene M. Ghobrial

Abstract The introduction of agents targeting multiple myeloma (MM), an incurable cancer of plasma cells (PC), in the context of the bone marrow (BM) microenvironment has radically changed the prognosis of the disease. Expected overall survival of patients has increased from 2-3 years in the 1950s to the currently estimated 7-8 years. The uncovering of the crucial pathogenetic role of the BM niche in nurturing MM cells by promoting survival, proliferation and drug resistance has been instrumental in designing more effective treatments. Historically, the stigmata of MM has been the presence of multiple foci of bone disease, herein the nomenclature "multiple". Together with the production of a monoclonal immunoglobulin, the capacity of cancer cells to traffic between distant bone sites and home to the BM is the pathognomonic feature of MM. In this chapter we will review the molecular mechanisms sustaining MM trafficking and homing to the BM and the maladaptive interaction with the BM niche, with an emphasis on the development of novel drugs to target these processes.

G. Bianchi
Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA

I.M. Ghobrial (✉)
Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA
e-mail: Irene_Ghobrial@DFCI.HARVARD.EDU

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_12,

12.1 Homing of Antibody-Producing Cells to the Bone Marrow: The Role of SDF-1/CXCR4 Axis in Health and Disease

12.1.1 Ontogenesis of Long-Lived Plasma Cells

Multiple myeloma (MM) is a cancer of terminally differentiated plasma cells (PC) [1]. It accounts for over 10% of hematologic malignancy and 2% of annual cancer-related deaths [2, 3]. The American Cancer Society estimates that almost 22,000 new cases of MM will be diagnosed in the year 2012 and 10,700 MM-related deaths are expected for the same year [4]. Despite the introduction of several new effective anti-MM drugs over the last two decades, this disease remains incurable with an estimated overall survival of 7–8 years [5].

Antigenic profile studies suggest that early genetic mutations in MM occur at pre-B cell stage, but the final oncogenic process takes place in terminally differentiated PC [6]. More specifically, MM cells are the malignant counterpart of long-lived PC, a subset of antibody-producing, terminally differentiated B cells whose name reflects their prolonged life span [7]. This peculiar characteristic distinguishes them from the more abundant post-germinal center (GC), short-lived PC which undergo apoptosis after 3–7 days of intense, high-affinity antibody secretion [8–10]. A tight control over the life span of PC is essential to appropriately terminate antibody production, thus limiting the potential side effects of sustained immunoglobulin synthesis, including autoimmunity [11–13].

Long-lived PC are also a product of GC maturation but, differently from short-lived ones, they electively home to specialized BM niches where they survive for months to years and produce low titer, high-affinity antibodies, important for immunologic memory [14, 15].

Basic science has elucidated the molecular mechanisms underlying the prolong survival of long-lived PC in the BM. Researchers observed that apoptosis quickly ensues when these cells are cultured ex vivo, thus suggesting the presence of essential survival signals in the BM microenvironment. Indeed, programmed cell death can be delayed by supplementing growth media with interleukin 6 (IL-6) and stromal cell-derived factor 1 (SDF-1) [14, 16].

The latter is a chemokine constitutively expressed by bone marrow stroma cells (BMSC) whose function is crucial throughout the process of B lymphopoiesis. It is a growth factor for pre-B cells and an essential chemoattractant in the process of homing and retention to the BM of mature B cells and PC [17–19]. The activity of SDF-1 (also known as CXCL-12) is mediated via binding to its cognate receptor CXCR4, a G protein coupled receptor widely expressed along different stages of B cell differentiation [20, 21]. At the pre-B cell level, CXCR4 guides localization to specialized BM niches; in mature B cells, it is essential for appropriate organization of GC; finally it mediates long-lived PC homing to the BM [22]. In the dynamic process of chemokine rearrangement, CXCR4 was shown to be progressively upregulated in B cell lineage during the process of differentiation to PC, accounting for

increased sensitivity to SDF-1 of terminally differentiated B lymphocytes [23, 24]. SDF-1 and CXCR4 are linked in an exclusive, univocal relationship as demonstrated by the reproduction of an identical, lethal phenotype in both Cxcr4 and Cxcl12 gene-deleted mice. Knockout animals for either one of these genes present with impaired myelopoiesis, especially involving the B cell lineage, and defective cardiovascular and neuronal development [25–27].

Mice genetically engineered to selectively lack expression of CXCR4 in the B cell lineage showed delayed, but not absent, recruitment and accumulation of PC in the BM, thus underlying both the importance of SDF-1/CXCR4 axis in PC homing and the redundancy of signaling pathways mediating it [28]. On the basis of these observations, it was speculated that two independent processes are responsible for the ontogenesis of long-lived PC: one dependent on CXCR4/SDF-1 for BM homing of post-GC PC, and a second mechanism of BM recruitment, GC- and CXCR4/SDF-1-independent, with terminal differentiation of B cells to long-lived PC in situ.

12.1.2 Multiple Myeloma Cell Recruitment and Retention to the Bone Marrow

Circulating, malignant PC are present in more than 70% of newly diagnosed MM patients, outlining the capacity of these cells to recirculate from peripheral blood to BM and vice versa [29, 30]. The complex process leading to the recruitment of MM cells to specialized BM niches is known as homing [31]. This nomenclature synthesized a series of events required for MM cells to extravasate and localize to the BM, including tenacious adhesion to endothelial cells (EC); crossing of the EC basement membrane; degradation of the extracellular matrix (ECM); and finally binding to pro-survival elements in the BM.

An intact SDF-1/CXCR4 axis is essential for MM cells homing to the BM and survival [32, 33]. As their normal counterpart, MM cells express high level of CXCR4 [34]. Upon binding with SDF-1, the latter mediates adhesion to vascular cell adhesion protein 1 (VCAM-1); ECM components, including fibronectin; and EC, via expression of $\alpha 4\beta 1$ [35]. Flow cytometry of primary patient cells showed CXCR4 to be expressed in 70% of MM cells of BM origin and 100% of MM cells obtained from peripheral blood or extramedullary sites [36]. Interestingly, the SDF-1 level was significantly higher in the BM milieu of patients with MM compared to healthy donors [35].

As the interaction of MM cells with the BM microenvironment is crucial for their survival, proliferation, and drug resistance, recent years have witnessed a growing interest in understanding the mechanisms underlying SDF-1-mediated recruitment of MM cells to the BM.

SDF-1 induces relocalization and polarization of CXCR4, followed by pseudopodia formation, eventually leading to chemotaxis of MM cells toward the source of SDF-1 [35]. In vitro, high concentrations of SDF-1 cause internalization of CXCR4, thus suggesting a negative feedback loop between ligand and receptor and explaining

the higher surface expression of CXCR4 in circulating MM cells compared to BM-extracted MM cells. Importantly, pharmacologic inhibition of CXCR4/SDF-1 binding, via anti-CXCR4 monoclonal antibody (mAb) or CXCR4 knockdown, abolished SDF-1-induced migration, confirming the univocal relationship between SDF-1 and CXCR4 [35]. In MM, the survival pathways mediated by phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) are downstream of CXCR4/SDF-1 as proved by their inactivation after CXCR4 inhibition. Moreover, MAP/ERK was shown to be downstream of PI3K, as inhibitors of the latter, but not of the former, blocked both signaling pathways.

Downstream of SDF-1 is also the small GTPase RalB that is necessary, but not sufficient, to drive SDF-1-induced chemotaxis [37]. Differently from other cell types, RalB activation in MM is independent of cytosolic kinases Lyn/Syk, phospholipase C (PLC), Bruton's tyrosine kinase (Btk), PI3K and Ras, and it has been suggested to be downstream of the β-arrestin and p38 pathway [38, 39].

Beyond RalB, two members of the Rho guanosine triphosphatase (GTPase) family, RhoA and Rac1, are mediators of MM adhesion and SDF-1-induced chemotaxis [40]. Gene expression profiling and flow cytometry studies proved RhoA and Rac1 to be expressed at a significantly higher level in MM cell lines and primary cells from MM patients, when compared to BM PC from healthy individuals [41]. Rho-associated protein kinase (ROCK) is downstream of RhoA and key to the homing process, by mediating interaction of MM cells with EC and ECM components, and adhesion to BMSC via a very late antigen 4 (VLA 4)/VCAM-1-dependent and leukocyte function-associated antigen 1/intracellular adhesion molecule 1 (LFA-1/ICAM-1)-independent mechanism [42–44].

Confocal microscopy studies in the presence of specific ROCK or Rac1 pharmacologic inhibitors showed SDF-1-induced actin polymerization and polarization to be heavily dependent on ROCK, while Rac1 blockade had a less prominent effect on the former and did not affect the latter. Moreover, ROCK, but not Rac1, inhibition resulted in increased dephosphorylation of myosin light chain (MLC), a key molecule in the regulation of contraction and relaxation of actin [45]. Altogether these results suggest that the RhoA/ROCK pathway rather than the Rac1 pathway is primarily responsible for SDF-1-dependent chemotaxis in MM cells in vitro. However, in vivo, ROCK and Rac1 proved to play an analogous role in MM cell homing, as pharmacologic inhibition of either one in a murine model caused a comparable delay in MM cell extravasation and BM engraftment [41].

12.1.3 SDF-1/CXCR4 and Their Downstream Effectors as Potential Therapeutic Targets in Multiple Myeloma

MM cell interaction with the BM microenvironment contributes to cancer cell survival, proliferation, and drug resistance (see Sect. 2) and inhibition of this maladaptive loop is an appealing therapeutic strategy in MM. The CXCR4/SDF-1 axis was the natural, primary target of such therapies, in consideration of its pivotal role in MM cell homing.

In vitro, AMD3100 (plerixafor, AnorMED, Toronto, ON, Canada), a selective, reversible bicyclam antagonist of SDF-1, sensitizes MM cells to novel and standard anti-MM agents such as bortezomib, melphalan, doxorubicin, and dexamethasone [35, 46]. Treatment of MM cells with the proteasome inhibitor (PI) bortezomib was shown to induce phosphorylation of Akt, suggesting a potential mechanism of resistance [47]. In coculture systems with BMSC, combinatory treatment of AMD3100 and bortezomib results in decreased phosphorylation of Akt compared to cells treated with the PI alone, providing a potential, molecular mechanism for the enhanced effectiveness of bortezomib in combination with plerixafor.

In mouse models, treatment with AMD3100 significantly reduced homing of MM cells to the BM niche compared to untreated animals. Homing and retention of hematopoietic stem cells (HSC) within the BM is also dependent on SDF-1/CXCR4 and the therapeutic use of AMD3100 raised concern in the scientific community for potential bone marrow suppression and toxicity to hematopoietic cell progenitors. Interestingly, in murine models, mobilization of HSC and MM cells after injection of AMD3100 followed different kinetics with cancer cells regressing later compared to HSC. This observation suggests that a timely administration of chemotherapy after AMD3100 could spare HSC and exquisitely targets MM cells [46].

The presence of circulating malignant cells in MM patients is a negative prognostic factor for overall survival [30]. As AMD3100 works primarily by mobilizing MM cells out of their survival BM niche and into the bloodstream, there is a reasonable concern that such treatment could increase disease aggressiveness and precipitate distant metastatic seeding. However, preclinical data are encouraging: mice treated with AMD3100 show neither more aggressive course of disease nor increase extramedullary engraftment of MM cells. Moreover, MM cells mobilized outside the BM by AMD3100 largely undergo apoptosis while in the blood stream upon bortezomib treatment, with no evidence of increased toxicity against HSC [46].

AMD3100 has been successfully used in phases I, II, and III clinical studies to aid mobilization of CD34 positive cells in patients undergoing harvesting for autologous bone marrow transplantation [48, 49]. No significant early or delayed toxicity, or inadequate HSC engraftment, was noticed with the use of AMD3100 alone or in combination with G-CSF [50–54]. Indeed, the addition of AMD3100 to G-CSF was proven to successfully mobilize CD34 positive cells in patients with hematologic malignancies, including MM, who had previously failed mobilization with cytokines alone [55].

Noticeably, the use of AMD3100 was not associated with significantly increased mobilization of MM cells to the peripheral blood or with tumor contamination of the CD34-positive apheresis products [56]. In the context of the data reported by Azab et al., this observation could be explained by the differential kinetics of BM egress for MM cells versus HSC.

A phase I/II clinical trial of plerixafor in combination with bortezomib is currently open for patients with relapsed/refractory MM.

T140 is a 14-amino acid, CXCR4 antagonist derived from a naturally occurring horseshoe crab–produced molecule. In comparative studies, T140 acts as a pure inverse agonist of CXCR4, while AMD3100 shows early and transient, weak

partial agonist activity, followed by sustained antagonism [57, 58]. These different pharmacodynamic properties reflect in a temporary, pro-proliferative and pro-survival effect of AMD3100, but not T140 [59]. Based on these observations, T140 could be more effective and safer when compared to AMD3100, prompting clinical evaluation in solid tumors. T140 is currently not undergoing clinical trials in hematologic malignancies.

The ROCK inhibitor Y27632 and the Rac1 inhibitor NSC23766 have been successfully used in vitro and in animal models to study the mechanisms underlying MM homing and trafficking. They have not been evaluated clinically, yet.

12.2 The Pathogenetic Role of the Bone Marrow Microenvironment

12.2.1 The Downstream Effects of Bone Marrow Stroma Cells–Multiple Myeloma Cells Interaction

The bone marrow microenvironment is composed by a cellular and a noncellular compartment. The former includes BMSC, EC, HSC, and hematopoietic progenitors, adipocytes, osteoblasts, and osteoclasts; the latter is the pool of ECM proteins such as laminin, fibronectin, and collagen [60, 61]. A definitive, relevant pathogenetic role for the BM microenvironment in MM has been recognized in recent years [62]. The binding of MM cells to BM cells causes autocrine and paracrine production of molecules involved in survival, proliferation, and neoangiogenesis such as IL-6, SDF-1, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin-like growth factor 1 (IGF-1), tumor necrosis factor α (TNF-α), and transforming growth factor β (TGF-β) [63–65]. This bidirectional positive feedback loop between MM cells and BM niche is sustained by the induction of several signaling pathways, including nuclear factor kB (NF-kB), MEK/ERK, Janus kinase/signal transducers and activator of transcription 3 (JNK/STAT3) and PI3K/AKT [16, 34, 66]. The downstream effects of their activation include proliferation (i.e., upregulation of cyclin D1; IL-6); survival (i.e., induction of antiapoptotic, multi-dimers Bcl proteins); neoangiogenesis (i.e., VEGF); BM homing and trafficking (i.e., ICAM-1, MMP-9, and MMP-1); and disruption of normal bone architecture via stimulation of osteoclastogenesis and inhibition of osteoblast differentiation [67–70].

Importantly, adhesion of MM cells to BMSC causes drug resistance via at least two distinct mechanisms: cell adhesion-mediated drug resistance (CAM-DR), which requires direct cell to cell contact; and via release of cytokines, including IL-6, in a contact-independent process [71–73].

Several adhesion molecules mediate MM-BMSC interaction: CD44, VLA 4 and 5, LFA-1, ICAM-1, VCAM-1, and syndecan-1, also known as CD138. The latter is a transmembrane proteoglycan strongly expressed by terminally differentiated PC and

MM cells that mediates adherence to ECM via binding of type I collagen. CD138-type I collagen interaction triggers expression of matrix metalloproteinase 1 (MMP-1), which has been shown to be crucial for ECM reabsorption and tumor invasion [67, 74, 75].

Disruption of the normally balanced osteoclast and osteoblast activity is a prominent feature of MM that not only guarantees cancer survival and proliferation but also determines major MM-related morbidity including pathologic fractures and hypercalcemia [76, 77]. MM bone microarchitecture is characterized by increased number and activity of osteoclasts and impaired new bone deposition due to suppression of osteoblast function [78, 79]. Several humoral factors are involved in supporting osteoclastogenesis and impairing osteoblast activity, including IL-6, receptor activator of NF-κB ligand (RANKL), B-cell activating factor (BAFF), VEGF, and dickkopf (DKK)-1 [80, 81]. A relative excess of the pro-osteoclastogenic RANKL in face of decreased osteoprotegerin (OPG), a RANKL decoy receptor, is at the base of myeloma bone disease pathogenesis and has been proven to be a predictive factor for bone disease in MM patients [79, 82, 83].

12.2.2 Pharmacologic Targets in Multiple Myeloma–Bone Marrow Niche Interaction

The maladaptive nature of MM cell–BM niche interaction and its central pathogenetic role in MM has been increasingly recognized in recent years thanks to growing insights from basic and translational research. Indeed, it is now widely recognized that in order to be effective, anti-MM drugs need to overcome the nurturing support of BMSC and be effective in the context of the BM microenvironment. The novel anti-MM agents, bortezomib, thalidomide, and lenalidomide, have been specifically proved active against MM while in the context of the BM niche.

The therapeutic application of mAb has provided the unique opportunity to design specific therapies to target MM cell–BM interaction while sparing normal tissues. MAb effectiveness relies both on the specificity of the target and on several mechanisms of toxicity such as antibody-dependent cell-mediated cytotoxicity (ADCC); complement-dependent cytotoxicity (CDC); and direct induction of apoptosis or growth arrest [84].

Flow cytometry, immunohistochemistry, and ELISA techniques have been instrumental in designing mAb for the treatment of MM by identifying surface molecules highly expressed by MM cells, such as CS1, CD38, CD40, CD74, and CD70; or soluble pro-survival, pro-angiogenic, and growth factors present in abundance in the BM milieu (IL-6, VEGF, EGFR, DKK-1, and RANKL) [85]. Most of these targets have been validated in preclinical models and are currently been evaluated as single agent or in combinatory regimens with lenalidomide and bortezomib in clinical trials [85]. IL-6 has a prominent pathogenetic role in MM, being not only a growth factor for cancer cells but also an important mediator of dexamethasone resistance [86]. In vitro, the chimeric mAb against IL-6, siltuximab (CNTO 328), enhanced the anti-MM activity of both standard (melphalan, dex) and novel (bortezomib) therapies [87].

In combination with these drugs, it was shown to cause apoptosis via activation of both caspase 8 and 9 [88]. Siltuximab proved to be safe in phase I and II clinical trials and is currently undergoing phase III clinical evaluation in combination with dex and bortezomib in relapsed, refractory MM patients [89, 90].

Elotuzumab (HuLuc6) is a mAb directed against CS1, a transmembrane glycoprotein highly expressed on MM cells that contributes to the interaction between cancerous cells and BM niche [91]. In vitro, elotuzumab triggers natural killer (NK)-mediated ADCC against MM cells and synergize with bortezomib, thalidomide, and lenalidomide. In clinical trials, elotuzumab was only modestly active against MM as a single agent, while it showed promising results in combination with lenalidomide and bortezomib. Several clinical trials of multidrug regimens containing elotuzumab and bortezomib (or lenalidomide) are currently enrolling newly diagnosed and refractory/relapsing MM patients [92–94].

Given their specificity for target cells, MAb have also been used as a vehicle to selectively deliver highly cytotoxic drugs to cancer in an attempt to improve effectiveness and reduce systemic toxicity. This is the case also for MM where an anti-CD-138 antibody conjugated to maytansinoid, toxic derivatives of the microtubule inhibitor maytansine, showed encouraging anti-MM effect in animal models [95].

Myeloma-related bone disease is a cause of major morbidity and poor quality of life. Most recently, the unmatched activity of OC over OB has been suggested to also play a role in promoting disease progression and MM cell proliferation [80]. Indeed, in mouse models, treatment with OPG not only decreased bone reabsorption but also suppressed MM proliferation, suggesting a bidirectional pro-survival loop between OC and cancer cells [96].

Bisphosphonates are the standard of care of myeloma bone disease due to their pro-apoptotic and inhibitory effect against OC and have been historically considered supportive therapy [80]. More recently, a direct anti-MM activity of these compounds has been advocated on the base of direct cytotoxicity of nitrogen-containing bisphosphonates [zoledronic acid (ZA) and pamidronate] against MM cells in preclinical models as well as the evidence of a survival advantage in patients with breast cancer receiving ZA [97–99].

A randomized, prospective, controlled phase III clinical trial proved prolonged overall survival and progression-free survival in MM patients treated with ZA compared to patients receiving clodronate (an orally available bisphosphonate), providing the first clinical evidence of direct anti-cancer activity of ZA [100].

Several mAb have been developed to target bone remodeling with the aim of either increasing OB activity or inhibiting OC function. The molecular target of the mAb BHQ880 is Dickkopf −1 (DKK-1), an inhibitor of the WNT/β-catenin signaling pathway which directly suppresses OB function, including production of OPG, resulting in an overall favorable, pro-OC balance. In vitro, BHQ880 promotes OB differentiation and activity and results in indirect inhibition of tumor growth [101]. It is currently in phase I/II clinical evaluation in combination with ZA in patients with refractory/relapsed MM. Denosumab (AMG165), a RANKL-blocking antibody, proved to be an effective treatment of cancer-related bone disease in MM and other

malignancies, even in patients refractory to bisphosphonates, and is currently in phase III clinical trial [102, 103].

In preclinical studies, CCR1 inhibitor MLN3897, anti-B cell activating factor (BAFF) MAb and anti-activin MAb (RAP-011 and ACE-011) oppose OC–MM interaction and support OB function, thus providing the rational for their clinical evaluation [80, 85].

12.3 Trafficking of Multiple Myeloma Cells

12.3.1 The Role of Metallo- and Non-Metalloproteinases

The presence of multiple foci of bone disease is a hallmark feature of MM. Myeloma cells are capable of migrating from one BM site to another via blood circulation, a complex, multistep process collectively known as trafficking. First, cancer cells need to lose their bonds to cellular and acellular elements in the BM niche; then they need to migrate through ECM, subendothelial space, and basement membrane; and finally move across the endothelium lining to reach the vascular lumen. In order to extravasate and invade a metastatic site, circulating MM cells need to recognize chemotactic signaling; adhere tenaciously to the EC surface and perform the same sequence of migratory steps, but in inverse order.

Metalloproteinases (MMP), a family of multidomain, zinc-dependent, endopeptidases, have been implicated in both physiological (leukocyte extravasation and tissue remodeling in inflammation or wound repair) and pathological (metastatic seeding and tissue invasion) processes [104–106]. MMP exist as integral transmembrane proteins, namely membrane-type MMP (MT-MMP), or as zymogenic enzymes, which are secreted inactive and acquire function through in situ cleavage [107–109]. The activity of MMP is modulated tightly at the transcriptional, translational, and post-translational level. Tissue inhibitors of MMP (TIMP) are a family of small molecular weight proteins whose function is to reversibly inhibit MMP via stereotactic interaction [110]. The fine equilibrium between MMP activity and their counter-regulatory mechanisms defines the overall proteolytic outcome [111–114].

When compared to normal PC, primary MM cells and BMSC from MM patients produce an excess of several MMP: MMP-1, MMP-2, MMP-8, MMP-9, MMP-13, and MTI-MMP [67, 74]. In vitro, migration of MM cells through Matrigel, an ECM extract that mimics the composition of basement membrane, is inhibited by blocking SDF-1/CXCR4 interaction or by using a broad MMP inhibitor (GM6001) [115]. MMP-9 is secreted by MM cells in response to SDF-1 exposure and participates in migration through ECM and EC. Its blockade, either pharmacological (via anti MMP-9 antibodies) or via TIMP-1, a naturally produced, specific inhibitor, significantly abrogates SDF-1-induced MM migration. MMP-9 is believed to favor metastatic spread of MM cells also by releasing matrix-bond VEGF-A, thus allowing it to bind its receptor and initiate the neoangiogenetic cascade [116].

SDF-1 induces expression of MT1-MMP and its discrete polarization along a unique site on the cell membrane of MM cells. Inhibition studies with blocking mAb and pharmacologic inhibitors showed MT1-MMP to mediate migration of MM cells through Matrigel upon SFD-1 exposure.

MMP have also been implicated in the recruitment of OC at the sites of bone remodeling, suggesting a causative role for these enzymes in bone disease and osteolytic lesions [117].

Mouse models have been used to further investigate the putative role of MMP in MM trafficking and disease progression. In 5T2MM-bearing mice, treatment with SC-964, a broad MMP inhibitor, reduced significantly disease burden, osteolytic lesions, and neoangiogenesis, suggesting a pivotal role for MMP in MM pathogenesis [118].

Enzymes other than MMP have recently been implicated in the process of tumor cell migration and metastasization. Heparanase is an endo-β-D-glucuronidase highly expressed in both solid and hematologic cancers, including MM, that has raised significant interest for its role in cell dissemination and metastasis [119]. Its primarily function is cleavage of heparan sulfate side chains from proteoglycans (i.e., syndecan-1), an activity that has been associated with metastasization, neoangiogenesis, and inflammation. Specifically in MM, high level of heparanase was shown to reduce nuclear syndecan-1 and increase histone acetyltransferase enzymes activity, resulting in increased protein transcription and aggressive cancer phenotype [120]. In consideration of these functions, heparanase appears to be an appealing drug target in MM.

12.3.2 Integrins in Multiple Myeloma

A wide array of integrins, including α4, α5, αv, β1, β2, β3, and β7, are expressed by MM cells and involved in the processes of homing and trafficking. Their activity is regulated both by binding with ECM, and BM milieu cytokines and chemokines, as well as by inside-out signaling [70, 121][122, 123]

VLA-4 (α4β1), VLA-5 (α5β1), and αvβ3 were the first integrins to be recognized for their role in mediating MM homing to the BM, malignant cell trafficking, and drug resistance [71, 124–127]. More recently, a role for integrin β7 in MM pathogenesis has been proposed. Integrin β7 is a negative prognostic factor for overall survival and predictor of poor response to standard and novel therapy in MM patients [128]. It was first identified as a downstream target of the oncogene C-MAF, which is overexpressed in almost half of MM patients either via genetic rearrangement (translocation t(14;16)) or paracrine stimulation along the BAFF/APRIL/TACI (B-cell-activating factor/a proliferation-inducing ligand/transmembrane activator and calcium modulator and cyclophilin ligand interactor) axis. In MM cells, knock down or mAb-mediated inhibition of integrin β7 caused decreased adhesion to fibronectin, E-cadherin, and BMSC; impaired invasion of Matrigel; and diminished migration along an SDF-1 gradient; resulting in increased sensitivity to standard

and novel chemotherapy agents such as melphalan and bortezomib. Moreover, integrin β7 was shown to induce VEGF, macrophage inflammatory protein 1β (MIP-1β), and interleukin 1β (IL-1β) secretion in MM and BMSC, suggesting a role in mediating neo-angiogenesis and osteoclast activity [128]. Consistent with in vitro data, knock down of integrin β7 in a mouse model caused delayed homing and tumor engraftment and decreased neoangiogenesis.

12.3.3 The Challenges of Pharmacological Inhibition of Multiple Myeloma Trafficking

The pivotal role of MMP in cell trafficking, neoangiogenesis, and maladaptive bone remodeling makes them a promising pharmacologic target in MM and solid malignancies [129, 130]. However, the clinical validation of anti-MMP molecules has been largely unsuccessful, underscoring the difficulties in understanding the complex process of trafficking and the specific role of individual enzymes, as well as the limitations of currently available disease models.

As an example, Neovastat (AE-941), a purified extract of shark cartilage, showed promising anti-neoplastic and anti-nonangiogenic activity in vitro via inhibition of EC, fibroblast, muscular skeleton, and cancer cell growth as well as direct blockade of MMP-2, -9, -12, and VEGFR-2 [131]. However, a phase II clinical trial in relapsed-refractory MM showed no significant activity of Neovastat as a single agent [132]. There are currently no MMP inhibitors in clinical development in MM.

SST0001, a heparin-derivative with no anticoagulation properties, inhibits heparanase activity in MM preclinical models. It has shown promising results in animal studies, causing direct inhibition of cancer growth and neoangiogenesis. The anti-MM activity of SST0001 is predicated on the downstream effects of heparanase inhibition: downregulation of HGF, VEGF, and MMP-9 and decreased syndecan-1 shedding [133]. Moreover, when used in combination with dexamethasone (dex), SST0001 proved effective in arresting tumor growth in xenograft mouse models injected with dex-resistant MM cells. On the basis of these promising preclinical results, SST001 is currently undergoing evaluation for entering clinical trials in MM.

Integrins are a challenging pharmacologic target given their pleiotropic expression and major role in several physiologic activities. Rather, targeting molecules involved in neoangiogenesis, a largely cancer-specific process, seems to be a more selective approach. In particular, the VEGF/VEGFR pathway has been considered an appealing pharmacologic target, given its overexpression in MM and key pathogenetic role. However, similarly to MMP inhibitors, development of safe and effective inhibitors of the VEGF/VEGFR axis has been challenging.

ZD6474 is an orally bioavailable, selective inhibitor of VEGFR and EGFR kinase activity. While theoretically promising based on preclinical data, it failed to demonstrate activity in refractory/relapsing MM patients as monotherapy in phase II study [134]. XL999, a small molecule inhibitor of VEGFR, PDGFR, and FGFR, showed promising activity in phase I clinical trials, but a phase II trial in MM was

terminated due to significant cardiac toxicity and its development has since been discontinued.

The VEGF-blocking mAb bevacizumab is currently undergoing phase I clinical studies in advanced hematologic and solid malignancies. In refractory/relapsing MM patients, it has been evaluated in combination with bortezomib alone or as part of a bortezomib-containing multidrug regimen [135, 136].

12.4 A Role for Hypoxia in Promoting Multiple Myeloma Cell Trafficking

12.4.1 Pathogenetic Functions of HIF-1 and 2 in Multiple Myeloma

The discovery that hypoxemic cells are present in the vast majority of tumor beds has been a landmark in the history of medicine [137]. The role of hypoxia in determining chemo- and radiation-resistance as well as promoting neoangiogenesis and metastasis has opened a new era in oncology and a promising field in pharmacology. The bone marrow is physiologically characterized by a lower oxygen tension compared to other tissues, and MM therefore exemplifies the paradigm of hypoxemic tumor.

Hypoxia inducible factors (HIF) are a family of transcription factors that sense hypoxia and trigger a signaling pathway aimed at restoring homeostasis in oxygen tension via neoangiogenesis and cell migration [138]. HIF-1α and 2α are the most studied members of this family and have been thought to be largely overlapping in their proto-oncogenic function [139–141]. HIF-1α and 2α appear to play a pathogenic role in MM [142, 143]. Immunohistochemistry studies found both molecules to be frequently overexpressed in primary MM cells and their expression to correlate with VEGF production and neoangiogenesis. HIF-1α overexpression was also associated with increased MM cell survival and trafficking, representing a poor prognostic factor for patients [143–145].

Among the mechanisms advocated for HIF1α and HIF2α oncogenic effect in MM is the induction of CXCR4, resulting in increased cancer cell trafficking and metastatic spread [146, 147]. HIF-2α was found to be a positive regulator of aberrant expression of SDF-1 in MM cell lines, providing a second mechanism to support MM cell migration and homing [148].

Recent data also showed HIF-1α expression to increase proportionally with MM cell proliferation and BM invasion. In return, HIF-1α downregulates E-cadherin expression in a process known as epithelial–mesenchymal transition, and upregulates CXCR4, with the net result of decreased MM cell adhesion to BMSC and increased cancer cell egression from BM and metastatic seeding [149].

12.4.2 Turning Hypoxia Against Cancer: A New Pharmacologic Option for the Cure of Multiple Myeloma?

There is growing interest in suppressing HIF function in MM to reduce neoangiogenesis and metastatic spread. In preclinical studies, blockade of HIF-1α activity via either RNA silencing or small molecules inhibitors resulted in effective anti-MM activity and provides the rational for clinical validation of this novel target [150–152]. Currently, there are no HIF inhibitors in clinical development in MM, although clinical trials are ongoing in solid tumors.

For instance, EZN-2968 is an antisense oligonucleotide that specifically binds HIF-1α mRNA, once internalized by cells, thus abrogating its transcription [153]. Phase I study in metastatic, advanced solid malignancies proved a safe toxicity profile and phase II studies are eagerly awaited.

A different approach to exploit the hypoxemic cancer microenvironment against MM was recently developed. TH-302 is a prodrug designed to be activated to the highly cytotoxic bromo-isophosphoramide mustard only in the presence of hypoxia, with the rational of limiting toxicity to the normo-perfused healthy tissues [154]. In murine models of MM, TH-302 effectively reduced paraprotein and microvessel density, suggesting an effect on both MM proliferation and neoangiogenesis. Based on these results, a phase I/II clinical trial of TH-302, alone or in combination with bortezomib, in patients with refractory MM is currently recruiting (clinicaltrials.gov).

12.5 The Importance and Limitations of Animal Models to Study Multiple Myeloma Trafficking and Homing

Homing and trafficking of MM cells proved to be a challenging process to study due to difficulties in reproducing the disease in animal models and in following cancer cell circulation in real time. Moreover, mouse models are intrinsically flawed due to inherent differences between murine and human bone marrow microenvironment [155]. Several animals have been developed in the past two decades with the intent of overcoming these challenges.

The first murine model available to study MM trafficking was the 5T2MM. The latter is a murine myeloma cell line derived from elderly C57Bl/KaLwRij mice that reproduces a phenotype similar to MM when injected intravenously in syngenic, young mice [156]. These cells produce a monoclonal Ig protein, home to the BM, where they proliferate and induce neoangiogenesis, and are capable or recirculating, thus causing the typical pattern of multiple osteolytic lesions [96, 157].

The 5T2MM mice were used to evaluate the effect of MMP inhibition in MM cell homing, survival, and proliferation. This animal model proved adequate for assessing BM homing, neo-angiogenesis, and disease burden; however, it had two major limitations: first, MM cell trafficking could not be monitored real time, and second, both cancer and BMSC studied were murine and not human [118].

The plasmacytoma mouse model attempted to address this latter caveat by subcutaneously injecting human MM cells in immunodeficient mice. This *escamotage* allowed to directly study human cancer cells in an animal model [158]. The major limitation of this model is the heterotopic localization of MM cells, outside their natural BM microenvironment, thus being of no utility for the study of MM homing and trafficking [159].

Intravenous injection of MM cells in severe combined immunodeficient/non-obese diabetic (SCID/NOD) resulted in MM cell homing to different BM sites, with a considerable improvement compared to the plasmacytoma model. However, similarly to the 5T2MM model, the circulation and homing of MM cell could not be monitored real time.

The development and biomedical application of optic techniques based on luminescence and fluorescence filled this gap and provided the unique opportunity to visualize real-time cancer cell trafficking [160].

The kinetic of homing of malignant cells in irradiated, SCID/NOD mice injected intravenously with luciferase positive (Luc+) or lentiviral-transfected GFP-luciferin-Neo MM.1S cells could be followed at close interval by means of whole body, real-time bioluminescence [161, 162]. These mouse models were used to study the effect of the SDF-1 inhibitor AMD3100 on MM cell trafficking and homing as well as the role of hypoxia in promoting MM cell egression from the BM and trafficking [35, 46, 149].

The use of GFP-tagged MM cells had the advantage of allowing high-resolution images throughout the intact animal skull and direct visualization of the bone marrow niche via fluorescence confocal microscopy. Apoptosis of cancer cells could also be detected via in vivo flow cytometry without the need of blood draws or mice sacrifice [46, 163].

Given the amount of information provided by the SCID mouse model injected with fluorescent MM.1S, these mice have been widely used to study the role of several molecules, such as integrin β7, ROCK and Rac1 in MM cell proliferation, homing and trafficking [41, 128].

A caveat for these animals is the lack of a human BM microenvironment as a dock for human MM cell homing. It is difficult to anticipate the inter-species variability and differences in the structure and function of the BM niches and consequently the impact of this limitation on the data gathered from these animals.

Current efforts have been put in developing a mouse model that could overcome this limitation.

12.6 Conclusions and Remarks

In the past two decades, basic and translational science has provided a critical mass of information regarding the biology of MM and the prominent pathogenetic role of the BM microenvironment. Homing of MM cells to the BM and trafficking among distant bone niches have been recognized as crucial in the progression of the disease. The complexity of the molecular mechanisms of MM adhesion and recirculation,

the pathogenic role of signaling pathways also involved in physiologic functions, such as hematopoiesis and inflammation, as well as the limitations of currently available animal models, have made the development of tolerable, effective drugs targeting homing and trafficking extremely challenging.

A deeper understanding of the importance of the different molecules participating in these processes is eagerly awaited in order to develop more selective, tolerable drugs to help cure MM patients.

References

1. Anderson KC, Carrasco RD (2011) Pathogenesis of myeloma. Annu Rev Pathol 6:249–274. doi:10.1146/annurev-pathol-011110-130249
2. Kyle RA, Rajkumar SV (2007) Epidemiology of the plasma-cell disorders. Best Pract Res Clin Haematol 20(4):637–664. doi:10.1016/j.beha.2007.08.001
3. Kyle RA, Rajkumar SV (2008) Multiple myeloma. Blood 111(6):2962–2972. doi:10.1182/blood-2007-10-078022
4. American Cancer Society (2012). Cancer Facts and Figures 2012. American Cancer Society. Atlanta, GA:
5. Kumar SK, Rajkumar SV, Dispenzieri A, Lacy MQ, Hayman SR, Buadi FK, Zeldenrust SR, Dingli D, Russell SJ, Lust JA, Greipp PR, Kyle RA, Gertz MA (2008) Improved survival in multiple myeloma and the impact of novel therapies. Blood 111(5):2516–2520. doi:10.1182/blood-2007-10-116129
6. Bergsagel PL, Chesi M, Nardini E, Brents LA, Kirby SL, Kuehl WM (1996) Promiscuous translocations into immunoglobulin heavy chain switch regions in multiple myeloma. Proc Natl Acad Sci USA 93(24):13931–13936
7. Manz RA, Arce S, Cassese G, Hauser AE, Hiepe F, Radbruch A (2002) Humoral immunity and long-lived plasma cells. Curr Opin Immunol 14(4):517–521
8. Calame KL (2001) Plasma cells: finding new light at the end of B cell development. Nat Immunol 2(12):1103–1108. doi:10.1038/ni1201-1103
9. Calame KL, Lin KI, Tunyaplin C (2003) Regulatory mechanisms that determine the development and function of plasma cells. Annu Rev Immunol 21:205–230. doi:10.1146/annurev.immunol.21.120601.141138
10. Shapiro-Shelef M, Calame K (2005) Regulation of plasma-cell development. Nat Rev Immunol 5(3):230–242. doi:10.1038/nri1572
11. Cascio P, Oliva L, Cerruti F, Mariani E, Pasqualetto E, Cenci S, Sitia R (2008) Dampening Ab responses using proteasome inhibitors following in vivo B cell activation. Eur J Immunol 38(3):658–667. doi:10.1002/eji.200737743
12. Cenci S, Mezghrani A, Cascio P, Bianchi G, Cerruti F, Fra A, Lelouard H, Masciarelli S, Mattioli L, Oliva L, Orsi A, Pasqualetto E, Pierre P, Ruffato E, Tagliavacca L, Sitia R (2006) Progressively impaired proteasomal capacity during terminal plasma cell differentiation. EMBO J 25(5):1104–1113. doi:10.1038/sj.emboj.7601009
13. Neubert K, Meister S, Moser K, Weisel F, Maseda D, Amann K, Wiethe C, Winkler TH, Kalden JR, Manz RA, Voll RE (2008) The proteasome inhibitor bortezomib depletes plasma cells and protects mice with lupus-like disease from nephritis. Nat Med 14(7):748–755. doi:10.1038/nm1763
14. Minges Wols HA, Underhill GH, Kansas GS, Witte PL (2002) The role of bone marrow-derived stromal cells in the maintenance of plasma cell longevity. J Immunol 169(8):4213–4221
15. Moser K, Tokoyoda K, Radbruch A, MacLennan I, Manz RA (2006) Stromal niches, plasma cell differentiation and survival. Curr Opin Immunol 18(3):265–270. doi:10.1016/j.coi.2006.03.004

16. Cassese G, Arce S, Hauser AE, Lehnert K, Moewes B, Mostarac M, Muehlinghaus G, Szyska M, Radbruch A, Manz RA (2003) Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. J Immunol 171(4):1684–1690
17. Ansel KM, Cyster JG (2001) Chemokines in lymphopoiesis and lymphoid organ development. Curr Opin Immunol 13(2):172–179
18. Campbell DJ, Kim CH, Butcher EC (2003) Chemokines in the systemic organization of immunity. Immunol Rev 195:58–71
19. Tokoyoda K, Egawa T, Sugiyama T, Choi BI, Nagasawa T (2004) Cellular niches controlling B lymphocyte behavior within bone marrow during development. Immunity 20(6):707–718. doi:doi:10.1016/j.immuni.2004.05.001
20. Bleul CC, Fuhlbrigge RC, Casasnovas JM, Aiuti A, Springer TA (1996) A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). J Exp Med 184(3): 1101–1109
21. Nakayama T, Hieshima K, Izawa D, Tatsumi Y, Kanamaru A, Yoshie O (2003) Cutting edge: profile of chemokine receptor expression on human plasma cells accounts for their efficient recruitment to target tissues. J Immunol 170(3):1136–1140
22. Allen CD, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, Cyster JG (2004) Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. Nat Immunol 5(9):943–952. doi:10.1038/ni1100
23. Hargreaves DC, Hyman PL, Lu TT, Ngo VN, Bidgol A, Suzuki G, Zou YR, Littman DR, Cyster JG (2001) A coordinated change in chemokine responsiveness guides plasma cell movements. J Exp Med 194(1):45–56
24. Hauser AE, Debes GF, Arce S, Cassese G, Hamann A, Radbruch A, Manz RA (2002) Chemotactic responsiveness toward ligands for CXCR3 and CXCR4 is regulated on plasma blasts during the time course of a memory immune response. J Immunol 169(3):1277–1282
25. Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, Bronson RT, Springer TA (1998) Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc Natl Acad Sci USA 95(16):9448–9453
26. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, Yoshida N, Kikutani H, Kishimoto T (1996) Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature 382(6592):635–638. doi:10.1038/382635a0
27. Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR (1998) Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 393(6685):595–599. doi:10.1038/31269
28. Nie Y, Waite J, Brewer F, Sunshine MJ, Littman DR, Zou YR (2004) The role of CXCR4 in maintaining peripheral B cell compartments and humoral immunity. J Exp Med 200(9):1145–1156. doi:10.1084/jem.20041185
29. Billadeau D, Van Ness B, Kimlinger T, Kyle RA, Therneau TM, Greipp PR, Witzig TE (1996) Clonal circulating cells are common in plasma cell proliferative disorders: a comparison of monoclonal gammopathy of undetermined significance, smoldering multiple myeloma, and active myeloma. Blood 88(1):289–296
30. Nowakowski GS, Witzig TE, Dingli D, Tracz MJ, Gertz MA, Lacy MQ, Lust JA, Dispenzieri A, Greipp PR, Kyle RA, Rajkumar SV (2005) Circulating plasma cells detected by flow cytometry as a predictor of survival in 302 patients with newly diagnosed multiple myeloma. Blood 106(7):2276–2279. doi:10.1182/blood-2005-05-1858
31. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC (2007) Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. Nat Rev Cancer 7(8):585–598. doi:10.1038/nrc2189
32. Aggarwal R, Ghobrial IM, Roodman GD (2006) Chemokines in multiple myeloma. Exp Hematol 34(10):1289–1295. doi:10.1016/j.exphem.2006.06.017
33. Moller C, Stromberg T, Juremalm M, Nilsson K, Nilsson G (2003) Expression and function of chemokine receptors in human multiple myeloma. Leukemia 17(1):203–210. doi:10.1038/sj.leu.2402717, 2402717 [pii]

34. Hideshima T, Chauhan D, Hayashi T, Podar K, Akiyama M, Gupta D, Richardson P, Munshi N, Anderson KC (2002) The biological sequelae of stromal cell-derived factor-1alpha in multiple myeloma. Mol Cancer Ther 1(7):539–544
35. Alsayed Y, Ngo H, Runnels J, Leleu X, Singha UK, Pitsillides CM, Spencer JA, Kimlinger T, Ghobrial JM, Jia X, Lu G, Timm M, Kumar A, Cote D, Veilleux I, Hedin KE, Roodman GD, Witzig TE, Kung AL, Hideshima T, Anderson KC, Lin CP, Ghobrial IM (2007) Mechanisms of regulation of CXCR4/SDF-1 (CXCL12)-dependent migration and homing in multiple myeloma. Blood 109(7):2708–2717. doi:10.1182/blood-2006-07-035857
36. Trentin L, Miorin M, Facco M, Baesso I, Carraro S, Cabrelle A, Maschio N, Bortoli M, Binotto G, Piazza F, Adami F, Zambello R, Agostini C, Semenzato G (2007) Multiple myeloma plasma cells show different chemokine receptor profiles at sites of disease activity. Br J Haematol 138(5):594–602. doi:10.1111/j.1365-2141.2007.06686.x
37. de Gorter DJ, Reijmers RM, Beuling EA, Naber HP, Kuil A, Kersten MJ, Pals ST, Spaargaren M (2008) The small GTPase Ral mediates SDF-1-induced migration of B cells and multiple myeloma cells. Blood 111(7):3364–3372. doi:10.1182/blood-2007-08-106583
38. Fong AM, Premont RT, Richardson RM, Yu YR, Lefkowitz RJ, Patel DD (2002) Defective lymphocyte chemotaxis in beta-arrestin2- and GRK6-deficient mice. Proc Natl Acad Sci USA 99(11):7478–7483. doi:10.1073/pnas.112198299
39. Sun Y, Cheng Z, Ma L, Pei G (2002) Beta-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation. J Biol Chem 277(51):49212–49219. doi:10.1074/jbc.M207294200
40. Etienne-Manneville S, Hall A (2002) Rho GTPases in cell biology. Nature 420(6916):629–635. doi:10.1038/nature01148
41. Azab AK, Azab F, Blotta S, Pitsillides CM, Thompson B, Runnels JM, Roccaro AM, Ngo HT, Melhem MR, Sacco A, Jia X, Anderson KC, Lin CP, Rollins BJ, Ghobrial IM (2009) RhoA and Rac1 GTPases play major and differential roles in stromal cell-derived factor-1-induced cell adhesion and chemotaxis in multiple myeloma. Blood 114(3):619–629. doi:10.1182/blood-2009-01-199281
42. Amano M, Fukata Y, Kaibuchi K (2000) Regulation and functions of Rho-associated kinase. Exp Cell Res 261(1):44–51. doi:10.1006/excr.2000.5046
43. Leung T, Chen XQ, Manser E, Lim L (1996) The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. Mol Cell Biol 16(10):5313–5327
44. Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K (1996) Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. EMBO J 15(9):2208–2216
45. Kamm KE, Stull JT (2011) Signaling to myosin regulatory light chain in sarcomeres. J Biol Chem 286(12):9941–9947. doi:10.1074/jbc.R110.198697
46. Azab AK, Runnels JM, Pitsillides C, Moreau AS, Azab F, Leleu X, Jia X, Wright R, Ospina B, Carlson AL, Alt C, Burwick N, Roccaro AM, Ngo HT, Farag M, Melhem MR, Sacco A, Munshi NC, Hideshima T, Rollins BJ, Anderson KC, Kung AL, Lin CP, Ghobrial IM (2009) CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy. Blood 113(18):4341–4351. doi:10.1182/blood-2008-10-186668
47. Hideshima T, Catley L, Yasui H, Ishitsuka K, Raje N, Mitsiades C, Podar K, Munshi NC, Chauhan D, Richardson PG, Anderson KC (2006) Perifosine, an oral bioactive novel alkylphospholipid, inhibits Akt and induces in vitro and in vivo cytotoxicity in human multiple myeloma cells. Blood 107(10):4053–4062. doi:10.1182/blood-2005-08-3434
48. Lapidot T, Petit I (2002) Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. Exp Hematol 30(9):973–981
49. Pelus LM, Horowitz D, Cooper SC, King AG (2002) Peripheral blood stem cell mobilization. A role for CXC chemokines. Crit Rev Oncol Hematol 43(3):257–275

50. Broxmeyer HE, Orschell CM, Clapp DW, Hangoc G, Cooper S, Plett PA, Liles WC, Li X, Graham-Evans B, Campbell TB, Calandra G, Bridger G, Dale DC, Srour EF (2005) Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. J Exp Med 201(8):1307–1318. doi:10.1084/jem.20041385
51. Devine SM, Flomenberg N, Vesole DH, Liesveld J, Weisdorf D, Badel K, Calandra G, DiPersio JF (2004) Rapid mobilization of CD34+ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-Hodgkin's lymphoma. J Clin Oncol 22(6):1095–1102. doi:10.1200/JCO.2004.07.131
52. Devine SM, Vij R, Rettig M, Todt L, McGlauchlen K, Fisher N, Devine H, Link DC, Calandra G, Bridger G, Westervelt P, Dipersio JF (2008) Rapid mobilization of functional donor hematopoietic cells without G-CSF using AMD3100, an antagonist of the CXCR4/SDF-1 interaction. Blood 112(4):990–998. doi:10.1182/blood-2007-12-130179
53. Flomenberg N, Devine SM, Dipersio JF, Liesveld JL, McCarty JM, Rowley SD, Vesole DH, Badel K, Calandra G (2005) The use of AMD3100 plus G-CSF for autologous hematopoietic progenitor cell mobilization is superior to G-CSF alone. Blood 106(5):1867–1874. doi:10.1182/blood-2005-02-0468
54. Liles WC, Rodger E, Broxmeyer HE, Dehner C, Badel K, Calandra G, Christensen J, Wood B, Price TH, Dale DC (2005) Augmented mobilization and collection of CD34+ hematopoietic cells from normal human volunteers stimulated with granulocyte-colony-stimulating factor by single-dose administration of AMD3100, a CXCR4 antagonist. Transfusion 45(3):295–300. doi:10.1111/j.1537-2995.2005.04222.x
55. Calandra G, McCarty J, McGuirk J, Tricot G, Crocker SA, Badel K, Grove B, Dye A, Bridger G (2008) AMD3100 plus G-CSF can successfully mobilize CD34+ cells from non-Hodgkin's lymphoma, Hodgkin's disease and multiple myeloma patients previously failing mobilization with chemotherapy and/or cytokine treatment: compassionate use data. Bone Marrow Transplant 41(4):331–338. doi:10.1038/sj.bmt.1705908
56. Fruehauf S, Ehninger G, Hubel K, Topaly J, Goldschmidt H, Ho AD, Muller S, Moos M, Badel K, Calandra G (2010) Mobilization of peripheral blood stem cells for autologous transplant in non-Hodgkin's lymphoma and multiple myeloma patients by plerixafor and G-CSF and detection of tumor cell mobilization by PCR in multiple myeloma patients. Bone Marrow Transplant 45(2):269–275. doi:10.1038/bmt.2009.142
57. Trent JO, Wang ZX, Murray JL, Shao W, Tamamura H, Fujii N, Peiper SC (2003) Lipid bilayer simulations of CXCR4 with inverse agonists and weak partial agonists. J Biol Chem 278(47):47136–47144. doi:10.1074/jbc.M307850200
58. Zhang WB, Navenot JM, Haribabu B, Tamamura H, Hiramatu K, Omagari A, Pei G, Manfredi JP, Fujii N, Broach JR, Peiper SC (2002) A point mutation that confers constitutive activity to CXCR4 reveals that T140 is an inverse agonist and that AMD3100 and ALX40-4C are weak partial agonists. J Biol Chem 277(27):24515–24521. doi:10.1074/jbc.M200889200
59. Kim HY, Hwang JY, Kim SW, Lee HJ, Yun HJ, Kim S, Jo DY (2010) The CXCR4 antagonist AMD3100 has dual effects on survival and proliferation of myeloma cells in vitro. Cancer Res Treat 42(4):225–234. doi:10.4143/crt.2010.42.4.225
60. Basak GW, Srivastava AS, Malhotra R, Carrier E (2009) Multiple myeloma bone marrow niche. Curr Pharm Biotechnol 10(3):345–346
61. Mitsiades CS, McMillin DW, Klippel S, Hideshima T, Chauhan D, Richardson PG, Munshi NC, Anderson KC (2007) The role of the bone marrow microenvironment in the pathophysiology of myeloma and its significance in the development of more effective therapies. Hematol Oncol Clin North Am 21(6):1007–1034 (vii–viii. doi:10.1016/j.hoc.2007.08.007)
62. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC (2007) Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. Nat Rev Cancer 7(8):585–598. doi:nrc2189 [pii] 10.1038/nrc2189
63. Caligaris-Cappio F, Gregoretti MG, Merico F, Gottardi D, Ghia P, Parvis G, Bergui L (1992) Bone marrow microenvironment and the progression of multiple myeloma. Leuk Lymphoma 8(1–2):15–22. doi:10.3109/10428199209049813

64. Hayashi T, Hideshima T, Nguyen AN, Munoz O, Podar K, Hamasaki M, Ishitsuka K, Yasui H, Richardson P, Chakravarty S, Murphy A, Chauhan D, Higgins LS, Anderson KC (2004) Transforming growth factor beta receptor I kinase inhibitor down-regulates cytokine secretion and multiple myeloma cell growth in the bone marrow microenvironment. Clin Cancer Res 10(22):7540–7546. doi:10.1158/1078-0432.CCR-04-0632
65. Podar K, Chauhan D, Anderson KC (2009) Bone marrow microenvironment and the identification of new targets for myeloma therapy. Leukemia 23(1):10–24. doi:10.1038/leu.2008.259
66. Chauhan D, Uchiyama H, Akbarali Y, Urashima M, Yamamoto K, Libermann TA, Anderson KC (1996) Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-kappa B. Blood 87(3):1104–1112, clinicaltrials.gov
67. Barille S, Akhoundi C, Collette M, Mellerin MP, Rapp MJ, Harousseau JL, Bataille R, Amiot M (1997) Metalloproteinases in multiple myeloma: production of matrix metalloproteinase-9 (MMP-9), activation of proMMP-2, and induction of MMP-1 by myeloma cells. Blood 90(4):1649–1655
68. Dankbar B, Padro T, Leo R, Feldmann B, Kropff M, Mesters RM, Serve H, Berdel WE, Kienast J (2000) Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interactions in multiple myeloma. Blood 95(8):2630–2636
69. Gupta D, Treon SP, Shima Y, Hideshima T, Podar K, Tai YT, Lin B, Lentzsch S, Davies FE, Chauhan D, Schlossman RL, Richardson P, Ralph P, Wu L, Payvandi F, Muller G, Stirling DI, Anderson KC (2001) Adherence of multiple myeloma cells to bone marrow stromal cells upregulates vascular endothelial growth factor secretion: therapeutic applications. Leukemia 15(12):1950–1961
70. Uchiyama H, Barut BA, Mohrbacher AF, Chauhan D, Anderson KC (1993) Adhesion of human myeloma-derived cell lines to bone marrow stromal cells stimulates interleukin-6 secretion. Blood 82(12):3712–3720
71. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS (1999) Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood 93(5):1658–1667
72. Damiano JS, Dalton WS (2000) Integrin-mediated drug resistance in multiple myeloma. Leuk Lymphoma 38(1–2):71–81. doi:10.3109/10428190009060320
73. Vincent T, Mechti N (2005) Extracellular matrix in bone marrow can mediate drug resistance in myeloma. Leuk Lymphoma 46(6):803–811. doi:10.1080/10428190500051448
74. Kelly T, Borset M, Abe E, Gaddy-Kurten D, Sanderson RD (2000) Matrix metalloproteinases in multiple myeloma. Leuk Lymphoma 37(3–4):273–281. doi:10.3109/10428190009089428
75. Zdzisinska B, Walter-Croneck A, Kandefer-Szerszen M (2008) Matrix metalloproteinases-1 and −2, and tissue inhibitor of metalloproteinase-2 production is abnormal in bone marrow stromal cells of multiple myeloma patients. Leuk Res 32(11):1763–1769. doi:10.1016/j.leukres.2008.04.001
76. Abe M, Hiura K, Wilde J, Shioyasono A, Moriyama K, Hashimoto T, Kido S, Oshima T, Shibata H, Ozaki S, Inoue D, Matsumoto T (2004) Osteoclasts enhance myeloma cell growth and survival via cell–cell contact: a vicious cycle between bone destruction and myeloma expansion. Blood 104(8):2484–2491. doi:10.1182/blood-2003-11-3839
77. Bataille R, Chappard D, Marcelli C, Dessauw P, Sany J, Baldet P, Alexandre C (1989) Mechanisms of bone destruction in multiple myeloma: the importance of an unbalanced process in determining the severity of lytic bone disease. J Clin Oncol 7(12):1909–1914
78. Ashcroft AJ, Davies FE, Morgan GJ (2003) Aetiology of bone disease and the role of bisphosphonates in multiple myeloma. Lancet Oncol 4(5):284–292
79. Giuliani N, Bataille R, Mancini C, Lazzaretti M, Barille S (2001) Myeloma cells induce imbalance in the osteoprotegerin/osteoprotegerin ligand system in the human bone marrow environment. Blood 98(13):3527–3533
80. Raje N, Roodman GD (2011) Advances in the biology and treatment of bone disease in multiple myeloma. Clin Cancer Res 17(6):1278–1286. doi:10.1158/1078-0432.CCR-10-1804

81. Tian E, Zhan F, Walker R, Rasmussen E, Ma Y, Barlogie B, Shaughnessy JD Jr (2003) The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. N Engl J Med 349(26):2483–2494. doi:10.1056/NEJMoa030847
82. Roux S, Meignin V, Quillard J, Meduri G, Guiochon-Mantel A, Fermand JP, Milgrom E, Mariette X (2002) RANK (receptor activator of nuclear factor-kappaB) and RANKL expression in multiple myeloma. Br J Haematol 117(1):86–92
83. Seidel C, Hjertner O, Abildgaard N, Heickendorff L, Hjorth M, Westin J, Nielsen JL, Hjorth-Hansen H, Waage A, Sundan A, Borset M (2001) Serum osteoprotegerin levels are reduced in patients with multiple myeloma with lytic bone disease. Blood 98(7):2269–2271
84. Shuptrine CW, Surana R, Weiner LM (2012) Monoclonal antibodies for the treatment of cancer. Semin Cancer Biol 22(1):3–13. doi:10.1016/j.semcancer.2011.12.009
85. Richardson PG, Lonial S, Jakubowiak AJ, Harousseau JL, Anderson KC (2011) Monoclonal antibodies in the treatment of multiple myeloma. Br J Haematol. doi:10.1111/j.1365-2141.2011.08790.x
86. Chauhan D, Hideshima T, Pandey P, Treon S, Teoh G, Raje N, Rosen S, Krett N, Husson H, Avraham S, Kharbanda S, Anderson KC (1999) RAFTK/PYK2-dependent and -independent apoptosis in multiple myeloma cells. Oncogene 18(48):6733–6740. doi:10.1038/sj.onc.1203082
87. Trikha M, Corringham R, Klein B, Rossi JF (2003) Targeted anti-interleukin-6 monoclonal antibody therapy for cancer: a review of the rationale and clinical evidence. Clin Cancer Res 9(13):4653–4665
88. Voorhees PM, Chen Q, Kuhn DJ, Small GW, Hunsucker SA, Strader JS, Corringham RE, Zaki MH, Nemeth JA, Orlowski RZ (2007) Inhibition of interleukin-6 signaling with CNTO 328 enhances the activity of bortezomib in preclinical models of multiple myeloma. Clin Cancer Res 13(21):6469–6478. doi:10.1158/1078-0432.CCR-07-1293
89. Hunsucker SA, Magarotto V, Kuhn DJ, Kornblau SM, Wang M, Weber DM, Thomas SK, Shah JJ, Voorhees PM, Xie H, Cornfeld M, Nemeth JA, Orlowski RZ (2011) Blockade of interleukin-6 signalling with siltuximab enhances melphalan cytotoxicity in preclinical models of multiple myeloma. Br J Haematol 152(5):579–592. doi:10.1111/j.1365-2141.2010.08533.x
90. Voorhees PM, Chen Q, Small GW, Kuhn DJ, Hunsucker SA, Nemeth JA, Orlowski RZ (2009) Targeted inhibition of interleukin-6 with CNTO 328 sensitizes pre-clinical models of multiple myeloma to dexamethasone-mediated cell death. Br J Haematol 145(4):481–490. doi:10.1111/j.1365-2141.2009.07647.x
91. Hsi ED, Steinle R, Balasa B, Szmania S, Draksharapu A, Shum BP, Huseni M, Powers D, Nanisetti A, Zhang Y, Rice AG, van Abbema A, Wong M, Liu G, Zhan F, Dillon M, Chen S, Rhodes S, Fuh F, Tsurushita N, Kumar S, Vexler V, Shaughnessy JD Jr, Barlogie B, van Rhee F, Hussein M, Afar DE, Williams MB (2008) CS1, a potential new therapeutic antibody target for the treatment of multiple myeloma. Clin Cancer Res 14(9):2775–2784. doi:10.1158/1078-0432.CCR-07-4246
92. Jakubowiak AJ, Benson DM, Bensinger W, Siegel DS, Zimmerman TM, Mohrbacher A, Richardson PG, Afar DE, Singhal AK, Anderson KC (2012) Phase I trial of Anti-CS1 monoclonal antibody elotuzumab in combination with bortezomib in the treatment of relapsed/refractory multiple myeloma. J Clin Oncol. doi:10.1200/JCO.2011.37.7069
93. Morgan G (2010) Future drug developments in multiple myeloma: an overview of novel lenalidomide-based combination therapies. Blood Rev 24(Suppl 1):S27–S32. doi:10.1016/S0268-960X(10)70006-0
94. van Rhee F, Szmania SM, Dillon M, van Abbema AM, Li X, Stone MK, Garg TK, Shi J, Moreno-Bost AM, Yun R, Balasa B, Ganguly B, Chao D, Rice AG, Zhan F, Shaughnessy JD Jr, Barlogie B, Yaccoby S, Afar DE (2009) Combinatorial efficacy of anti-CS1 monoclonal antibody elotuzumab (HuLuc63) and bortezomib against multiple myeloma. Mol Cancer Ther 8(9):2616–2624. doi:10.1158/1535-7163.MCT-09-0483
95. Ikeda H, Hideshima T, Fulciniti M, Lutz RJ, Yasui H, Okawa Y, Kiziltepe T, Vallet S, Pozzi S, Santo L, Perrone G, Tai YT, Cirstea D, Raje NS, Uherek C, Dalken B, Aigner S, Osterroth F, Munshi N, Richardson P, Anderson KC (2009) The monoclonal antibody nBT062 conjugated to cytotoxic Maytansinoids has selective cytotoxicity against CD138-positive multiple

myeloma cells in vitro and in vivo. Clin Cancer Res 15(12):4028–4037. doi:10.1158/1078-0432.CCR-08-2867
96. Vanderkerken K, Asosingh K, Croucher P, Van Camp B (2003) Multiple myeloma biology: lessons from the 5TMM models. Immunol Rev 194:196–206
97. Croucher PI, De Hendrik R, Perry MJ, Hijzen A, Shipman CM, Lippitt J, Green J, Van Marck E, Van Camp B, Vanderkerken K (2003) Zoledronic acid treatment of 5T2MM-bearing mice inhibits the development of myeloma bone disease: evidence for decreased osteolysis, tumor burden and angiogenesis, and increased survival. J Bone Miner Res 18(3):482–492. doi:10.1359/jbmr.2003.18.3.482
98. Gnant M, Mlineritsch B, Schippinger W, Luschin-Ebengreuth G, Postlberger S, Menzel C, Jakesz R, Seifert M, Hubalek M, Bjelic-Radisic V, Samonigg H, Tausch C, Eidtmann H, Steger G, Kwasny W, Dubsky P, Fridrik M, Fitzal F, Stierer M, Rucklinger E, Greil R, Marth C (2009) Endocrine therapy plus zoledronic acid in premenopausal breast cancer. N Engl J Med 360(7):679–691. doi:10.1056/NEJMoa0806285
99. Guenther A, Gordon S, Tiemann M, Burger R, Bakker F, Green JR, Baum W, Roelofs AJ, Rogers MJ, Gramatzki M (2010) The bisphosphonate zoledronic acid has antimyeloma activity in vivo by inhibition of protein prenylation. Int J Cancer 126(1):239–246. doi:10.1002/ijc.24758
100. Morgan GJ, Davies FE, Gregory WM, Cocks K, Bell SE, Szubert AJ, Navarro-Coy N, Drayson MT, Owen RG, Feyler S, Ashcroft AJ, Ross F, Byrne J, Roddie H, Rudin C, Cook G, Jackson GH, Child JA (2010) First-line treatment with zoledronic acid as compared with clodronic acid in multiple myeloma (MRC Myeloma IX): a randomised controlled trial. Lancet 376(9757):1989–1999. doi:10.1016/S0140-6736(10)62051-X
101. Fulciniti M, Tassone P, Hideshima T, Vallet S, Nanjappa P, Ettenberg SA, Shen Z, Patel N, Tai YT, Chauhan D, Mitsiades C, Prabhala R, Raje N, Anderson KC, Stover DR, Munshi NC (2009) Anti-DKK1 mAb (BHQ880) as a potential therapeutic agent for multiple myeloma. Blood 114(2):371–379. doi:10.1182/blood-2008-11-191577
102. Castellano D, Sepulveda JM, Garcia-Escobar I, Rodriguez-Antolin A, Sundlov A, Cortes-Funes H (2011) The role of RANK-ligand inhibition in cancer: the story of denosumab. Oncologist 16(2):136–145. doi:10.1634/theoncologist.2010-0154
103. Vij R, Horvath N, Spencer A, Taylor K, Vadhan-Raj S, Vescio R, Smith J, Qian Y, Yeh H, Jun S (2009) An open-label, phase 2 trial of denosumab in the treatment of relapsed or plateau-phase multiple myeloma. Am J Hematol 84(10):650–656. doi:10.1002/ajh.21509
104. Egeblad M, Werb Z (2002) New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2(3):161–174. doi:10.1038/nrc745
105. Gialeli C, Theocharis AD, Karamanos NK (2011) Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. FEBS J 278(1):16–27. doi:10.1111/j.1742-4658.2010.07919.x
106. Stefanidakis M, Koivunen E (2006) Cell-surface association between matrix metalloproteinases and integrins: role of the complexes in leukocyte migration and cancer progression. Blood 108(5):1441–1450. doi:10.1182/blood-2006-02-005363
107. McCawley LJ, Matrisian LM (2001) Matrix metalloproteinases: they're not just for matrix anymore! Curr Opin Cell Biol 13(5):534–540
108. Nagase H, Woessner JF Jr (1999) Matrix metalloproteinases. J Biol Chem 274(31):21491–21494
109. Stamenkovic I (2003) Extracellular matrix remodelling: the role of matrix metalloproteinases. J Pathol 200(4):448–464. doi:10.1002/path.1400
110. Bourboulia D, Stetler-Stevenson WG (2010) Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): Positive and negative regulators in tumor cell adhesion. Semin Cancer Biol 20(3):161–168. doi:10.1016/j.semcancer.2010.05.002
111. Cruz-Munoz W, Khokha R (2008) The role of tissue inhibitors of metalloproteinases in tumorigenesis and metastasis. Crit Rev Clin Lab Sci 45(3):291–338. doi:10.1080/10408360801973244
112. Hojilla CV, Mohammed FF, Khokha R (2003) Matrix metalloproteinases and their tissue inhibitors direct cell fate during cancer development. Br J Cancer 89(10):1817–1821. doi:10.1038/sj.bjc.6601327

113. Sternlicht MD, Werb Z (2001) How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 17:463–516. doi:10.1146/annurev.cellbio.17.1.463
114. Visse R, Nagase H (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 92(8):827–839. doi:10.1161/01.RES.0000070112.80711.3D
115. Parmo-Cabanas M, Molina-Ortiz I, Matias-Roman S, Garcia-Bernal D, Carvajal-Vergara X, Valle I, Pandiella A, Arroyo AG, Teixido J (2006) Role of metalloproteinases MMP-9 and MT1-MMP in CXCL12-promoted myeloma cell invasion across basement membranes. J Pathol 208(1):108–118. doi:10.1002/path.1876
116. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2(10):737–744. doi:10.1038/35036374
117. Sato T, Foged NT, Delaisse JM (1998) The migration of purified osteoclasts through collagen is inhibited by matrix metalloproteinase inhibitors. J Bone Miner Res 13(1):59–66
118. Van Valckenborgh E, Croucher PI, De Raeve H, Carron C, De Leenheer E, Blacher S, Devy L, Noel A, De Bruyne E, Asosingh K, Van Riet I, Van Camp B, Vanderkerken K (2004) Multifunctional role of matrix metalloproteinases in multiple myeloma: a study in the 5T2MM mouse model. Am J Pathol 165(3):869–878. doi:10.1016/S0002-9440(10)63349-4
119. Arvatz G, Shafat I, Levy-Adam F, Ilan N, Vlodavsky I (2011) The heparanase system and tumor metastasis: is heparanase the seed and soil? Cancer Metastasis Rev 30(2):253–268
120. Purushothaman A, Hurst DR, Pisano C, Mizumoto S, Sugahara K, Sanderson RD (2011) Heparanase-mediated loss of nuclear syndecan-1 enhances histone acetyltransferase (HAT) activity to promote expression of genes that drive an aggressive tumor phenotype. J Biol Chem 286(35):30377–30383
121. Podar K, Tai YT, Lin BK, Narsimhan RP, Sattler M, Kijima T, Salgia R, Gupta D, Chauhan D, Anderson KC (2002) Vascular endothelial growth factor-induced migration of multiple myeloma cells is associated with beta 1 integrin- and phosphatidylinositol 3-kinase-dependent PKC alpha activation. J Biol Chem 277(10):7875–7881. doi:10.1074/jbc.M109068200
122. Holt RU, Baykov V, Ro TB, Brabrand S, Waage A, Sundan A, Borset M (2005) Human myeloma cells adhere to fibronectin in response to hepatocyte growth factor. Haematologica 90(4):479–488
123. Hurt EM, Wiestner A, Rosenwald A, Shaffer AL, Campo E, Grogan T, Bergsagel PL, Kuehl WM, Staudt LM (2004) Overexpression of c-maf is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma. Cancer Cell 5(2):191–199
124. Pellat-Deceunynck C, Barille S, Puthier D, Rapp MJ, Harousseau JL, Bataille R, Amiot M (1995) Adhesion molecules on human myeloma cells: significant changes in expression related to malignancy, tumor spreading, and immortalization. Cancer Res 55(16):3647–3653
125. Sanz-Rodriguez F, Ruiz-Velasco N, Pascual-Salcedo D, Teixido J (1999) Characterization of VLA-4-dependent myeloma cell adhesion to fibronectin and VCAM-1. Br J Haematol 107(4):825–834
126. Uchiyama H, Barut BA, Chauhan D, Cannistra SA, Anderson KC (1992) Characterization of adhesion molecules on human myeloma cell lines. Blood 80(9):2306–2314
127. Van Riet I, De Waele M, Remels L, Lacor P, Schots R, Van Camp B (1991) Expression of cytoadhesion molecules (CD56, CD54, CD18 and CD29) by myeloma plasma cells. Br J Haematol 79(3):421–427
128. Neri P, Ren L, Azab AK, Brentnall M, Gratton K, Klimowicz AC, Lin C, Duggan P, Tassone P, Mansoor A, Stewart DA, Boise LH, Ghobrial IM, Bahlis NJ (2011) Integrin {beta}7-mediated regulation of multiple myeloma cell adhesion, migration, and invasion. Blood 117(23):6202–6213. doi:10.1182/blood-2010-06-292243
129. Folgueras AR, Pendas AM, Sanchez LM, Lopez-Otin C (2004) Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies. Int J Dev Biol 48(5–6):411–424. doi:10.1387/ijdb.041811af

130. Lopez-Otin C, Palavalli LH, Samuels Y (2009) Protective roles of matrix metalloproteinases: from mouse models to human cancer. Cell Cycle 8(22):3657–3662
131. Gingras D, Boivin D, Deckers C, Gendron S, Barthomeuf C, Beliveau R (2003) Neovastat–a novel antiangiogenic drug for cancer therapy. Anti-cancer Drugs 14(2):91–96
132. Overall CM, Kleifeld O (2006) Tumour microenvironment – opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. Nat Rev Cancer 6(3):227–239. doi:10.1038/nrc1821
133. Ritchie JP, Ramani VC, Ren Y, Naggi A, Torri G, Casu B, Penco S, Pisano C, Carminati P, Tortoreto M, Zunino F, Vlodavsky I, Sanderson RD, Yang Y (2011) SST0001, a chemically modified heparin, inhibits myeloma growth and angiogenesis via disruption of the heparanase/syndecan-1 axis. Clin Cancer Res 17(6):1382–1393
134. Kovacs MJ, Reece DE, Marcellus D, Meyer RM, Mathews S, Dong RP, Eisenhauer E (2006) A phase II study of ZD6474 (Zactima, a selective inhibitor of VEGFR and EGFR tyrosine kinase in patients with relapsed multiple myeloma–NCIC CTG IND.145. Invest New Drugs 24(6):529–535. doi:10.1007/s10637-006-9022-7
135. Attar-Schneider O, Drucker L, Zismanov V, Tartakover-Matalon S, Rashid G, Lishner M (2012) Bevacizumab attenuates major signaling cascades and eIF4E translation initiation factor in multiple myeloma cells. Lab Invest 92(2):178–190. doi:10.1038/labinvest.2011.162
136. Somlo G, Lashkari A, Bellamy W, Zimmerman TM, Tuscano JM, O'Donnell MR, Mohrbacher AF, Forman SJ, Frankel P, Chen HX, Doroshow JH, Gandara DR (2011) Phase II randomized trial of bevacizumab versus bevacizumab and thalidomide for relapsed/refractory multiple myeloma: a California Cancer Consortium trial. Br J Haematol 154(4):533–535. doi:10.1111/j.1365-2141.2011.08623.x
137. Kieran MW, Folkman J, Heymach J (2003) Angiogenesis inhibitors and hypoxia. Nat Med 9(9):1104 (author reply 1104–1105. doi:10.1038/nm0903-1104a)
138. Semenza GL (2012) Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy. Trends Pharmacol Sci. doi:10.1016/j.tips.2012.01.005
139. Colla S, Storti P, Donofrio G, Todoerti K, Bolzoni M, Lazzaretti M, Abeltino M, Ippolito L, Neri A, Ribatti D, Rizzoli V, Martella E, Giuliani N (2010) Low bone marrow oxygen tension and hypoxia-inducible factor-1alpha overexpression characterize patients with multiple myeloma: role on the transcriptional and proangiogenic profiles of CD138(+) cells. Leukemia 24(11):1967–1970. doi:leu2010193 [pii] 10.1038/leu.2010.193
140. Danet GH, Pan Y, Luongo JL, Bonnet DA, Simon MC (2003) Expansion of human SCID-repopulating cells under hypoxic conditions. J Clin Invest 112(1):126–135. doi:10.1172/JCI17669
141. Keith B, Johnson RS, Simon MC (2012) HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. Nat Rev Cancer 12(1):9–22. doi:10.1038/nrc3183
142. Asosingh K, De Raeve H, de Ridder M, Storme GA, Willems A, Van Riet I, Van Camp B, Vanderkerken K (2005) Role of the hypoxic bone marrow microenvironment in 5T2MM murine myeloma tumor progression. Haematologica 90(6):810–817
143. Martin SK, Diamond P, Gronthos S, Peet DJ, Zannettino AC (2011) The emerging role of hypoxia, HIF-1 and HIF-2 in multiple myeloma. Leukemia 25(10):1533–1542. doi:10.1038/leu.2011.122 leu2011122 [pii]
144. Colla S, Storti P, Donofrio G, Lazzaretti M, Bonomini S, Lunghi P, Crugnola M, Bolzoni M, Galla L, Ippolito L, Martella E, Sammarelli G, Craviotto L, Caramatti C, Mangoni M, Bonati A, Rizzoli V, Giuliani N (2008) Hypoxia and hypoxia inducible factor (HIF)-1 alpha in multiple myeloma: effect on the pro-angiogenic signature of myeloma cells and the bone marrow microenvironment. Blood 112(11):595–596
145. Hu Y, Kirito K, Yoshida K, Mitsumori T, Nakajima K, Nozaki Y, Hamanaka S, Nagashima T, Kunitama M, Sakoe K, Komatsu N (2009) Inhibition of hypoxia-inducible factor-1 function enhances the sensitivity of multiple myeloma cells to melphalan. Mol Cancer Ther 8(8):2329–2338. doi:1535-7163.MCT-09-0150 [pii] 10.1158/1535-7163.MCT-09-0150
146. Schioppa T, Uranchimeg B, Saccani A, Biswas SK, Doni A, Rapisarda A, Bernasconi S, Saccani S, Nebuloni M, Vago L, Mantovani A, Melillo G, Sica A (2003) Regulation of the

chemokine receptor CXCR4 by hypoxia. J Exp Med 198(9):1391–1402. doi:10.1084/jem.20030267
147. Staller P, Sulitkova J, Lisztwan J, Moch H, Oakeley EJ, Krek W (2003) Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. Nature 425(6955):307–311. doi:10.1038/nature01874
148. Martin SK, Diamond P, Williams SA, To LB, Peet DJ, Fujii N, Gronthos S, Harris AL, Zannettino AC (2010) Hypoxia-inducible factor-2 is a novel regulator of aberrant CXCL12 expression in multiple myeloma plasma cells. Haematologica 95(5):776–784. doi:10.3324/haematol.2009.015628
149. Azab AK, Hu J, Quang P, Azab F, Pitsillides C, Awwad R, Thompson B, Maiso P, Sun JD, Hart CP, Roccaro AM, Sacco A, Ngo HT, Lin CP, Kung AL, Carrasco RD, Vanderkerken K, Ghobrial IM (2012) Hypoxia promotes dissemination of multiple myeloma through acquisition of endothelial to mesenchymal transition-like features. Blood. doi:10.1182/blood-2011-09-380410
150. Colla S, Tagliaferri S, Morandi F, Lunghi P, Donofrio G, Martorana D, Mancini C, Lazzaretti M, Mazzera L, Ravanetti L, Bonomini S, Ferrari L, Miranda C, Ladetto M, Neri TM, Neri A, Greco A, Mangoni M, Bonati A, Rizzoli V, Giuliani N (2007) The new tumor-suppressor gene inhibitor of growth family member 4 (ING4) regulates the production of proangiogenic molecules by myeloma cells and suppresses hypoxia-inducible factor-1 alpha (HIF-1alpha) activity: involvement in myeloma-induced angiogenesis. Blood 110(13):4464–4475. doi:10.1182/blood-2007-02-074617
151. Perrone G, Borsi E, Terragna C, Durante S, Martello M, Aluigi M, Mancini M, Zamagni E, Tacchetti P, Brioli A, Pantani L, Zannetti BA, Martinelli G, Santucci MA, Baccarani M, Cavo M (2011) HIF 1 Alpha: a suitable target for multiple myeloma. Paper presented at the American Society of Hematology (ASH) annual meeting, San Diego, California, USA
152. Storti P, Airoldi I, Bolzoni M, Lazzaretti M, Guasco D, Agnelli L, Martella E, Mancini C, Neri A, Donofrio G, Giuliani N (2011) Hypoxia-inducible factor (HIF)-1α is a therapeutic target in myeloma-induced angiogenesis. Paper presented at the American Society of Hematology (ASH) Annual Meeting, San Diego, California, USA
153. Greenberger LM, Horak ID, Filpula D, Sapra P, Westergaard M, Frydenlund HF, Albaek C, Schroder H, Orum H (2008) A RNA antagonist of hypoxia-inducible factor-1alpha, EZN-2968, inhibits tumor cell growth. Mol Cancer Ther 7(11):3598–3608. doi:10.1158/1535-7163.MCT-08-0510
154. Hu J, Handisides DR, Van Valckenborgh E, De Raeve H, Menu E, Vande Broek I, Liu Q, Sun JD, Van Camp B, Hart CP, Vanderkerken K (2010) Targeting the multiple myeloma hypoxic niche with TH-302, a hypoxia-activated prodrug. Blood 116(9):1524–1527. doi:10.1182/blood-2010-02-269126
155. Mitsiades CS, Anderson KC, Carrasco DR (2007) Mouse models of human myeloma. Hematol Oncol Clin North Am 21(6):1051–1069 (viii. doi:10.1016/j.hoc.2007.08.003)
156. Vanderkerken K, De Raeve H, Goes E, Van Meirvenne S, Radl J, Van Riet I, Thielemans K, Van Camp B (1997) Organ involvement and phenotypic adhesion profile of 5T2 and 5T33 myeloma cells in the C57BL/KaLwRij mouse. Br J Cancer 76(4):451–460
157. Asosingh K, Radl J, Van Riet I, Van Camp B, Vanderkerken K (2000) The 5TMM series: a useful in vivo mouse model of human multiple myeloma. Hematol J 1(5):351–356. doi:10.1038/sj/thj/6200052
158. LeBlanc R, Catley LP, Hideshima T, Lentzsch S, Mitsiades CS, Mitsiades N, Neuberg D, Goloubeva O, Pien CS, Adams J, Gupta D, Richardson PG, Munshi NC, Anderson KC (2002) Proteasome inhibitor PS-341 inhibits human myeloma cell growth in vivo and prolongs survival in a murine model. Cancer Res 62(17):4996–5000
159. Pilarski LM, Hipperson G, Seeberger K, Pruski E, Coupland RW, Belch AR (2000) Myeloma progenitors in the blood of patients with aggressive or minimal disease: engraftment and self-renewal of primary human myeloma in the bone marrow of NOD SCID mice. Blood 95(3):1056–1065

160. Novak J, Georgakoudi I, Wei X, Prossin A, Lin CP (2004) In vivo flow cytometer for real-time detection and quantification of circulating cells. Opt Lett 29(1):77–79
161. Mitsiades CS, Mitsiades NS, Bronson RT, Chauhan D, Munshi N, Treon SP, Maxwell CA, Pilarski L, Hideshima T, Hoffman RM, Anderson KC (2003) Fluorescence imaging of multiple myeloma cells in a clinically relevant SCID/NOD in vivo model: biologic and clinical implications. Cancer Res 63(20):6689–6696
162. Mitsiades CS, Mitsiades NS, McMullan CJ, Poulaki V, Shringarpure R, Akiyama M, Hideshima T, Chauhan D, Joseph M, Libermann TA, Garcia-Echeverria C, Pearson MA, Hofmann F, Anderson KC, Kung AL (2004) Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. Cancer Cell 5(3):221–230
163. Sipkins DA, Wei X, Wu JW, Runnels JM, Cote D, Means TK, Luster AD, Scadden DT, Lin CP (2005) In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature 435(7044):969–973. doi:10.1038/nature03703

Chapter 13
Genes and Proteins of Myeloma Endothelial Cells to Search Specific Targets of the Tumor Vasculature

Domenico Ribatti and Angelo Vacca

Abstract Multiple myeloma (MM) mainly progresses in the bone marrow (BM). Therefore signals from BM microenvironment are thought to play a critical role in maintaining plasma cell growth, migration, and survival. Reciprocal positive and negative interactions between plasma cells and BM stromal cells, namely endothelial cells (ECs), ECs progenitor cells, hematopoietic stem cells, osteoblasts/osteoclasts, chondroclasts, fibroblasts, macrophages, and mast cells, are mediated by an array of cytokines, receptors, and adhesion molecules. BM neovascularization is a constant hallmark of MM, and goes hand in hand with progression until leukemic phase. MM neovessels form through angiogenesis and vasculogenesis, and are endowed with the overangiogenic phenotype of ECs (MMECs). Induction of the vascular phase in MM is sustained by angiogenic cytokines, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), platelet derived growth factor (PDGF) and metalloproteinases, secreted by the BM plasma cells, and overexpressed in MMECs. BM microenvironmental factors induce MMECs to become functionally different from MGUS ECs (MGECs), i.e., to be characterized by an overangiogenic phenotype, and be similar to transformed cells. In fact, MMECs down- or up-regulate some genes like tumor cells. The induced phenotypic and genotypic modifications of MMECs entail at least 22 different genes that are ivolved in specific pathways which

D. Ribatti, M.D.
Department of Basic Medical Sciences, Section of Human Anatomy and Histology, University of Bari Medical School, Policlinico – Piazza Giulio Cesare, 11, I-70124 Bari, Italy
e-mail: domenico.ribatti@uniba.it

A. Vacca, M.D. (✉)
Department of Internal Medicine and Clinical Oncology, University of Bari Medical School, Policlinico – Piazza Giulio Cesare, 11, I-70124 Bari, Italy
e-mail: a.vacca@dimo.uniba.it

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_13,

control apoptosis, extracellular matrix formation and bone remodeling, cell adhesion, angiogenesis, and cell proliferation. These alterations play an important role in MM progression and may represent new molecular markers for prognostic stratification of patients and prediction of the response to antiangiogenic drugs as well as new potential therapeutic targets.

13.1 Differences Existing Between Normal and Tumor Vasculature

Considerable differences exist between normal and tumor vasculature. Tumor blood vessels are irregular in size, shape, and branching pattern, lack the normal hierarchy, and do not display the recognizable features of arterioles, capillaries, and venules.

Endothelial cells (ECs) of mature, quiescent vessels are low proliferative and their estimated turnover times are measured in years, whereas those of tumor vessels are markedly dependent on growth factor for their survival. Tumor ECs proliferate 50–200 times faster than normal ECs [1]. Their constant proliferation rate in some regions of the tumor vasculature reflects the angiogenesis that accompanies an increase in tumor volume, whereas in other regions tumor ECs undergo apoptosis in parallel with tumor necrosis and vessel regression.

The tumor-associated endothelium is structurally defective. Discontinuities or gaps that allow hemorrhage and facilitate permeability are common features. ECs contacts are usually poorly differentiated and no complex contact structures exist. Defects in ECs barrier function, due to abnormal cell–cell junctions and other changes, exaggerate leakiness. This correlates with histological grade and malignant potential [2], and can be exploited in locating tumors by imaging contrast media and in the delivery of macromolecular therapeutics [3]. Furthermore, these defects result in extravasation of plasma proteins and even erythrocytes, and may facilitate the traffic of tumor cells into the bloodstream and the formation of metastases [4]. Leakiness has been attributed to highly active angiogenesis and microvascular remodeling, but its structural basis and mechanism are unclear. Intercellular gaps, transendothelial holes, vesiculo-vacuolar organelles (VVO), and ECs fenestrae are all present in the endothelium of tumor vessels [4].

Tumor ECs also differ from those of normal vessels in other ways, including the profile and level of cell adhesion molecule they express. They preferentially overexpress the cell-surface molecules integrin $\alpha v\beta 3$ and $\alpha v\beta 5$, E-selectin, endoglin, endosialin, and VEGF receptors (VEGFRs), all of which stimulate ECs adhesion and migration [5]. Vascular endothelial cadherin (VE-cadherin) is poorly expressed in tumor vessels. This results in their destabilization and may lead to abnormal remodeling.

13.2 Peptides Isolated Through Phage Display Libraries and Mass Spectrometry

Very large collections ("libraries") of antibodies or peptides expressed on the surface of filamentous phage particles and substractive selection approaches have been used to search for differentially expressed cell surface molecules.

Arap et al. [6], by injecting peptide libraries into tumor-bearing mice followed by repeated rounds of phage isolation and reinjection, were able to identify several peptides which could selectively home to tumor vessels. Selection of phage from the libraries in tumor-bearing mice yielded three peptide motifs capable of homing to tumor vasculature. These are an RGD (arginine-glycine-aspartic acid) motif embedded in a double-cyclic peptide (termed RGD-4C), an NGR (asparagine-glycine-arginine) motif, and a GSL (glycine-serin-leucine) motif. A panel of peptide motifs, including the sequences RGD-4C, NGR, CPRECES, and GSL, have been assembled that target the tumor blood vessels [7].

Oh et al. [8] labeled the luminal surface of blood vessels of tumor-bearing mice with silica, performed subcellular fractionation of the isolated tissues, and used mass spectrometry to identify specific protein expressed at the ECs surface. Annexin-1 was the most specific tumor ECs target identified by this approach.

13.3 Gene Expression Abnormalities in Tumor Endothelial Cells

The use of antibodies specific for ECs receptors coupled with immunomagnetic separation has allowed for the isolation of pure populations of ECs from normal and tumor tissues.

St Croix et al. [9], by using this technique followed by serial analysis of gene expression (SAGE), were the first to show that colorectal cancer ECs overexpress specific transcripts as a result of qualitative differences in gene profiling compared with ECs of the normal colorectal mucosa. Of 79 transcripts differentially expressed, 46 were at least tenfold more elevated compared with normal ECs, whereas 33 were expressed at lower levels. Similar expression patterns were found in tumor-associated ECs from metastatic lesions and other primary tumor sites. The transcripts expressed at higher level encoded matrix proteins, but most were of unknown function. Nine novel cell surface markers, the tumor endothelial markers or TEM (TEM-1–TEM-9), were also identified. TEM-1, TEM-7, and TEM-8 show single-pass transmembrane domains. TEM-5 is an orphan seven-pass transmembrane G protein coupled receptor (GPCR) with a long extracellular amino-terminal domain and belongs to the so-called adhesion family of GPRC. TEM-1, TEM-5, and TEM-8 showed strong tumor-endothelial expression, but were absent from normal tissues [10]. The molecular cloning of endosialin showed it to be identical to TEM-1 [11]. TEM-1 mRNA appeared to be markedly elevated in a variety of cancer types and localized

to tumor neovasculature [12–14]. TEM-1 was most highly expressed in high-grade primary and metastatic brain tumors, lower in benign tumors, and absent in normal tissue surrounding the tumor [14]. TEM-7 was elevated in the endothelium of a variety of human tumor types, including colon, breast, bladder, esophageal, and brain cancer [15, 16]. TEM-8 is an anthrax-toxin receptor; the binding of the toxin to TEM-8 expressed on tumor endothelium, followed by ECs death, might explain the antitumor activity of the toxin [17]. Antibodies that have been generated against TEM-8 demonstrated that the protein is overexpressed in the endothelium of a variety of tumor types [18].

Most of the differentially expressed genes were also found during luteal angiogenesis and wound healing, indicating that tumor angiogenesis uses the same signaling pathways as physiological angiogenesis.

Van Baijnun et al. [19] compared transcriptional profiles of angiogenic ECs isolated from colorectal cancer and normal mucosa, as well as from placenta, and identified 17 genes that were overexpressed in tumor ECs but not in angiogenic ECs of the normal tissue.

Further studied in glioma [16, 20] and in invasive breast carcinoma [21, 22] have demonstrated a distinct gene expression pattern related to extracellular matrix and surface proteins characteristic of proliferating and migrating ECs, and pointed to specific roles for genes in driving tumor angiogenesis and progression of tumor cells.

13.4 Gene Expression Abnormalities in Multiple Myeloma Plasma Cells

Multiple myeloma (MM) is characterized by a profound genomic instability involving both numeric and structural genomic rearrangements [23].

Previous gene expression-profiling studies designed to identify genes involved in the initiation and progression of MM revealed that monoclonal gammopathies of undetermined significance (MGUS) and MM plasma cells can be distinguished from normal plasma cells [24, 25].

DNA microarrays enable to perform a molecular dissection of the diversity of MM and provides new molecular tools to investigate the pathogenesis of malignant plasma cells. Gene expression profiling has been used to define molecular characteristics of MM plasma cells according to Ig types/light chain subtypes and in relation to the degree of transformation [26–29].

Zhan et al. [26, 27] distinguished MM plasma cells from those of healthy donors based on the expression of approximately 120 of 6,800 genes analyzed and divided MM in four distinct molecular subgroups, MM-1–MM-4, with MM-1 being more similar to MGUS and MM-4 being related to MM cell lines. Genes distinguishing MM-4 from the other groups were related to cell proliferation.

Differentially expressed genes included oncogenes/tumor-suppressor genes (LAF4, RB1, and disabled homolog 2), cell-signaling genes [RAS family members, B-cell

signaling, and NF-kappa B (NFkB) genes], DNA-binding and transcription-factor genes (XBP1, zinc finger proteins, forkhead box, and ring finger proteins), and developmental genes (WNT and SHH pathways) [28].

The discovery of microRNA genes, encoding for a class of small noncoding RNAs involved in the regulation of cell cycle, survival and differentiation programs has added a further level of complexity to cancer cell biology. Roccaro et al. [30], performing microRNA expression profiling of MM cells vs. their normal cellular counterparts and validating data by RT-PCR, identified a MM-specific microRNA signature characterized by down-expression of microRNA-15a/16 and overexpression of microRNA-222/-221/-382/-181a/-181b. Moreover, they investigated the functional role of microRNA-15a and −16, and demonstrated that they regulate proliferation and growth of MM plasma cells in vitro and in vivo, by inhibiting AKT serine/threonine protein-kinase (AKT3), ribosomal-protein S6, MAP-kinases, NF-kB-activator MAPKIP3.

13.5 Angiogenesis in Multiple Myeloma

Under physiological conditions, angiogenesis depends on the balance of positive and negative angiogenic modulators within the vascular microenvironment. Tumor angiogenesis is linked to a switch in this balance, and mainly depends on the release by neoplastic cells of growth factors specific for ECs and able to stimulate the growth of the host's blood vessels [31].

Numerous clinical studies have shown that the degree of angiogenesis or the levels of angiogenic factors are correlated with the extent of stage of disease, prognosis or response to therapy [32]. Taken together these data strongly suggest that angiogenesis induction in solid and hematological tumors has a pathophysiology relevance for disease progression.

Angiogenesis is a constant hallmark of MM progression and has prognostic potential [33]. It is induced by plasma cells via angiogenic factors with the transition from MGUS to MM, and probably with loss of angiostatic activity on the part of MGUS. The pathophysiology of MM-induced angiogenesis is complex and involves both direct production of angiogenic cytokines by plasma cells and their induction within the microenvironment.

Bone marrow stroma cells (BMSCs) increase the concentration of angiogenic factors and matrix-degrading enzymes in the bone marrow microenvironment by direct secretion or by stimulation of MM plasma cells or ECs through paracrine interactions [34, 35]. BMSCs, osteoclasts, osteoblasts, and ECs secrete several factors, including VEGF, FGF-2, tumor necrosis factor alpha (TNF-α), IL-6, B-cell-activating factor, stromal cell-derived factor-1α (SDF-1α, also known as CXCL12), and various Notch family members, which are further upregulated by tumor cell adhesion to extracellular matrix proteins and/or BMSCs. Moreover, BMSCs and other accessory cells supporting MM plasma cell survival in the bone microenvironment constitute potential therapeutic targets [34, 35].

Finally, circulating ECs and endothelial precursor cells (EPCs) contribute to the neovascularization, and the presence of EPCs suggests that vasculogenesis (new vessel formation from EPCs) may also contribute to the full MM vascular tree [33].

13.6 Multiple Myeloma Endothelial Cells

In 2003, Vacca et al. [36] for the first time isolated ECs from bone marrow of patients with active Multiple myeloma endothelial cells (MMECs) and compared them with human umbilical vein ECs (HUVECs). MMECs showed a high expression of typical ECs markers (Tie2, VEGFR-2, FGFR-2, CD105-endoglin, and VE-cadherin), and high secretion of matrix metalloproteinases-2 and −9 (MMP-2 and MMP-9), and upregulation of angiogenic genes (VEGF, FGF-2, Gro-α chemokine, TGFβ, HIF-1α, ETS-1, and osteopontin). Moreover, MMECs expressed CD133 (AC133), a marker of the ECs progenitors that take part in vasculogenesis. MMECs showed intrinsic angiogenic ability in vitro and in vivo and ultrastructurally they are abnormal and were metabolically activated, like tumor ECs.

In 2005, Pellegrino et al. [37] further characterized MMECs and demonstrated that these cells secreted higher amounts of the CXC-chemokines CXCL8/intereleukin-8 (IL-8), CXCL11/interferon-inducible T cell alpha chemoattractant (I-TAC), CXCL12/stromal cell-derived factor (SDF)-1α, and CCL2/monocyte chemotactic protein (MCP)-1 than HUVECs. Paired plasma cells and several MM cell lines expressed cognate receptors of each chemokine to a variable extent.

More recently, Ria et al. [38] showed that hematopoietic stem and progenitor cells (HSPCs) of MM patients were able to differentiate into cells with ECs phenotype. In fact, HSPCs gradually lost CD133 expression and acquired VEGFR-2, factor VIII related antigen, and VE-cadherin expression.

Coluccia et al. [39] demonstrated that platelet-derived growth factor (PDGF)-BB/PDGF receptor beta (PDGFRβ) promoted the transcription of MMEC-proangiogenic factors, such as VEGF, FGF-2, and IL-8. Moreover, a prolonged exposure of MMECs to dasatinib, an oral bioactive PDGFRβ/SrcTK inhibitor, annulled their ability to respond to VEGF, preventing the expression of endogenous VEGF in a time-dependent manner, and the levels of secreted VEGF in the conditioned medium of MMECs in a dose-dependent manner.

13.7 Gene Expression Profiling in Multiple Myeloma Endothelial Cells

Hedvat et al. [40] compared gene expression profiling of MM cells, MM leukemia plasma cells, and extramedullary plasmacytoma cells, and identified several angiogenesis-related genes upregulated in extramedullary plasmacytoma cells.

Table 13.1 Genes differentially expressed in multiple myeloma endothelial cells (MMECs) as compared to endothelial cells of monoclonal gammopathy of undetermined significance (MGECs). Validation has been focused on BNIP3, IER3, SEPW1 CRYAB, SERPINF1, and SRPX genes, which have not been previously found to be functionally correlated to the overangiogenic phenotype of MMECs

Gene	Chromosoma	Protein	Function
BNIP 3	10q26.3	BCL2/adenovirus 19kDa interacting protein 3	Trascriptional factor regulated by hypoxia
IER 3	6p21.3	Immediate early response 3	Anti-apoptosis
SEPW1	19q13.3	Selenoprotein W, 1	omeostasys
CRYAB	11q22.3-q23.1	Crystallin, alpha B	Tubular morphogenesis
SERPINF1	17p13.1	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	Anti-angiogenesis
SRPX	Xp21.1	Sushi-repeat-containing protein, X-linked	Oncosuppressor

Munshi et al. [41] compared gene expression profiling of genetically identical twin samples and observed increased levels of expression of angiogenesis-related IL-8 and angiopoietin-1 (Ang-1) transcripts in MM cells as compared to healthy twin plasma cells.

Vacca et al. [42] demonstrated by means of DNA microarray and RT-PCR analysis an induction of VEGF, FGF-2, hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), and insulin-like growth factor binding protein-3 (IGFBP-3) in active MMECs, nonactive MMECs, and MGUS ECs (MGECs) over HUVECs. Exposure to thalidomide produced a significant downregulation of all genes in a dose-dependent fashion. The transcription factors Sp1 and NF-kB, both involved in angiogenesis, were also subjected to thalidomide modulation.

Hose et al. [43] evaluated the expression of 402 angiogenesis-associated genes in 466 gene-expressing profile, including normal bone marrow plasma cells, primary MM cells, and human MM cell lines. They demonstrated that although MM cells did not show a significantly higher median number of expressed pro- or antiangiogenic genes, 97% of MM cell samples expressed at least one of the following angiogenic factors—HGF, IL-15, Ang, APRIL, CTGF, or transforming growth factor alpha (TGF-α)—while the expression level of antiangiogenic genes remained constant.

Ria et al. [44] have identified gene differentially expressed in MMECs as compared to MGECs (Table 13.1). Deregulated genes were mostly involved in

extracellular matrix formation and bone remodeling, cell adhesion, chemotaxis, angiogenesis, resistance to apoptosis, and cell-cycle regulation. Validation was focused on BNIP3, IER3, SEPW1 CRYAB, SERPINF1, and SRPX genes, which were not previously found to be functionally correlated to the overangiogenic phenotype of MMECs.

In the apoptosis-related genes, BNPI3 belongs to the Bcl-2 family and is induced by hypoxia-inducible factor-1α (HIF1-α) [45]. HIF-1α is upregulated in the overangiogenic ECs isolated from patients with active MM at diagnosis, relapse, or leukemic phase [36], thus promoting the expression of VEGF [45]. Moreover, BNIP3 behaves as an antiapoptotic gene in MMECs, because BNIP3-small interfering RNA ECs increased apoptosis and decreased growth [44]. IER3 is a member of the "immediate early response gene" family induced by the antiapoptotic factor NF-kB in response to the TNF-α and ligand-mediated FAS, which acts as an antiapoptotic and a stress-inducible gene, playing an important role in cell survival under stress condition [46]. IER3 has been reported to be overexpressed in MM plasma cells [47] and small interfering RNA-silenced IER3 expression reduced cell proliferation and induced apoptosis in MMECs [44].

In the angiogenesis-related genes, SERPIN1, a serine protease inhibitor of angiogenesis [48], through Fas/Fas ligand-mediated apoptosis [49], was downregulated in MMECs [44].

In the cell proliferation and homeostasis genes, SEPW1, a gene with antioxidant function [50], was upregulated in MMECs and small interfering RNA-silenced expression of the gene inhibited MMECs adhesion and angiogenic activity [44].

In the signal transduction, cell-cycle regulation genes, DIRAS3, which negatively regulates cell growth and is associated with disease progression in breast and ovary carcinoma [51], was downregulated in MMECs [44].

A comparison between the 366 well-characterized genes resulting from the HUVECs vs. MMECs supervised gene expression profiling analysis [44] (Fig. 13.1) with the genes previously reported as differentially expressed by these two ECs types by means of a 96-gene cDNA array [36] indicates that, among 36 genes previously described, eight genes are differentially expressed at high stringency: the isoform 7 of the FGF, the VEGF isoforms VEGF-A and VEGF-C, fibronectin-1 and thrombospondin-2 were upregulated in MMECs, whereas the endothelial differentiating factor 1, CD105, and CD31 were downregulated [44].

Roccaro et al. [30] demonstrated that microRNA-15a/-16, which are downregulated in MM plasma cells as compared to normal cells, exert an antiangiogenic activity, reducing VEGF secretion from MM cells at the protein level, thereby reducing MM plasma cell proangiogenic activity on ECs and these data were further confirmed in vivo in the chorioallantoic membrane assay. Therefore, the antiangiogenic role of microRNA-15a and -16 may contribute, at least in part, to their anti-MM activity.

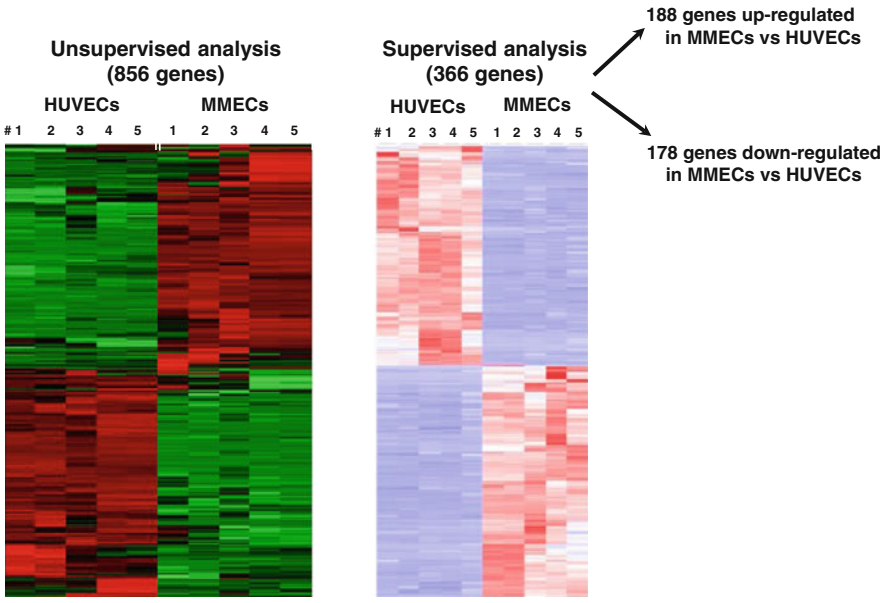


Fig. 13.1 Unsupervised and supervised analysis of gene expression profiles from human umbilical vein endothelial cells (HUVECs) and multiple myeloma endothelial cells (MMECs). HUVECs are different from MMECs for 856 genes in an unsupervised analysis and 366 genes in the supervised analysis: 188 are up-regulated, while 178 are down-regulated in MMECs as compared to HUVECs

13.8 Conclusions and Perspectives

Gene-expressing profile analysis in MM clearly evidences that bone marrow plasma cells express an excess of proangiogenic over antiangiogenic genes. Aberrant expression of proangiogenic genes by MMECs further increase the angiogenic stimulus already induced by tumor plasma cells (Fig. 13.2). However, it is conceivable that also microenvironmental factors, such as hypoxia, inflammation, expression of multiple cytokines and growth factors, regulating tumor-associated angiogenesis, may display unstable, heterogeneous, and progressive characteristics to an extent comparable with the instability of the cancer cell genome. In addition, those factors may have genetic causes and consequences, such as increased expression of oncogenes, loss of tumor suppressor genes. This reciprocal interrelationship and heterogeneity may translate into site- and stage-specific changes in the regulation of bone marrow angiogenesis and to changes in the proliferation and antiapoptotic potential of MM tumor plasma cells.

ECs lining tumor blood vessels express several cell surface markers that are absent in quiescent blood vessels. Accumulating knowledge of a number of cell surface tumor ECs markers indicates that they may be expressed across a broad variety of tumor types leading to the development of new, specific tumor ECs targets.

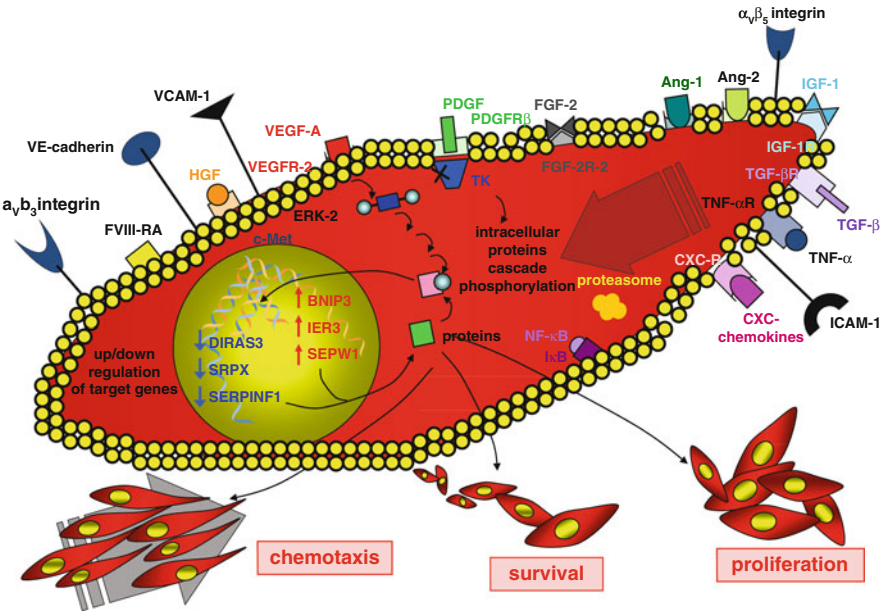


Fig. 13.2 Schematic overview of the complex interplay between different endothelial cell receptors, angiogenic cytokines, chemokines, and different genes involved in the characterization of the angiogenic phenotype of multiple myeloma endothelial cells (MMECs)

Antigenic determinants that are selectively and constitutively expressed on the tumor vasculature include endoglin, VEGF receptors, αν integrins, the fibronectin EDB domain, and prostate-specific membrane antigen (PSMA) [52].

VEGF and its receptors are upregulated in tumor microenvironment, leading to a high concentration of occupied receptors on tumor vascular endothelium. VEGF-receptor complexes were shown to be a specific target on tumor endothelium for antibodies in vivo. Targeting VEGF or its receptors with monoclonal antibodies (such as bevacizumab/Avastin) or small molecule inhibitors of VEGFR tyrosine kinase has confirmed the anticancer activity of these agents [53]. Fusion proteins and chemical conjugates of VEGF and diphtheria toxin or gelonin induced tumor regression in mice [52].

Ligand-directed vascular targeting can be accomplished by antibodies, specific peptides, or growth factors complexed with immunomodulatory, procoagulant or cytotoxic molecules [52]. Advances in proteomics and gene expression technology provide us a plethora of potential new targets. Moreover, it may be possible to use phage display techniques on tumor patient specimens with the aim to develop novel ligand-targeted liposomal chemotherapeutic strategies based on the selective targeting of other novel molecular markers expressed on the surface of tumor endothelial cells [54].

Acknowledgments This work was supported by Associazione Italiana per la Ricerca sul Cancro AIRC (National and Regional Funds) Milan, Fondazione Italiana per la Lotta al Neuroblastoma, Genoa, MIUR – FIRB 2001, PRIN 2005, and PRIN 2007, Rome, and Fondazione Cassa di Risparmio di Puglia, Bari, Italy. European Union Seventh Framework Programme (FPT7/2007–2013) under grant agreement n° 278570 to DR and n° 278706 to AV.

References

1. Vermeulen PB, Verhoeven D, Hubens G, Van Marck E, Goovaerts G, Huyghe M, De Bruijn EA, Van Oosterom AT, Dirix LY (1995) Microvessels density, endothelial cell proliferation and tumor cell proliferation in human colorectal adenocarcinomas. Ann Oncol 6:59–64
2. Daldrup H, Shames DM, Wendland M, Okuhata Y, Link TM, Rosenau W, Lu Y, Brasch RC (1998) Correlation of dynamic contrast-enhanced MR imaging with histologic tumor grade: comparison of macromolecular and small-molecular contrast media. Am J Roentgenol 171:941–949
3. Mc Donald DM, Choyke PL (2003) Imaging of angiogenesis: from microscope to clinic. Nat Med 9:713–725
4. Dvorak HF, Nagy JA, Dvorak JT, Dvorak AM (1988) Identification and characterization of the blood vessel of solid tumors that are leaky to circulating macromolecules. Am J Pathol 133:95–109
5. Magnussen A, Kasman IM, Norberg S, Baluk P, Murray R, McDonald DM (2005) Rapid access of antibodies to α5β1 integrin overexpressed on the luminal surface of tumor blood vessels. Cancer Res 65:2712–2721
6. Arap W, Pasqualini R, Ruoslahti E (1998) Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 279:377–380
7. Kolonin MG, Pasqualini R, Arap W (2001) Molecular addressed in blood vessel as targets for therapy. Curr Opin Chem Biol 5:308–313
8. Oh P, Li Y, Yu J, Durr E, Krasinska KM, Carver LA, Testa JE, Schnitzer JE (2004) Substractive proteomic mapping of the endothelial surface in lung and solid tumours for tissue specific therapy. Nature 429:629–635
9. St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, Kinzler KW (2000) Genes expressed in human tumor endothelium. Science 289:1197–1202
10. Carson-Walter EB, Watkins DN, Nanda A, Vogelstein B, Kinzler KW, St Croix B (2001) Cell surface tumor endothelial markers are conserved in mice and humans. Cancer Res 61:6649–6665
11. Christian S, Ahorn H, Novatchkova M, Garin-Chesa P, Park JE, Weber G, Eisenhaber F, Rettig WJ, Lenter MC (2001) Molecular cloning and characterization of endosialin, a C-type lectin-like cell surface receptor of tumor endothelium. J Biol Chem 276:7408–7414
12. Rettig WJ, Garin-Chesa P, Healey JH, Su SL, Jaffe EA, Old LJ (1992) Idemtification of endosialin, a cell syrface glycoprotein of vascular endothelial cells in human cancer. Proc Natl Acad Sci USA 89:10832–10836
13. Rupp C, Dolznig H, Puri C, Sommergruber W, Kerjaschki D, Rettig WJ, Garin-Chesa P (2006) Mouse endosialin, a C-type lectin-like cell surface receptor: expression during embryonic development and induction in experimental cancer neoangiogenesis. Cancer Immunol 6:10
14. Brady J, Neal J, Sadakar N, Gasque P (2004) Human endosialin (tumor endothelial marker 1) is abundantly expressed in highly malignant and invasive brian tumors. J Neuropathol Exp Neurol 63:1274–1283
15. Nanda A, Buckhaults P, Seaman S, Agrawal N, Boutin P, Shankara S, Nacht M, Teicher B, Stampfl J, Singh S, Vogelstein B, Kinzler KW, St Croix B (2004) Identification of a binding-patner for the endothelial cell surface proteins TEM7 and TEM7R. Cancer Res 64:8507–8511
16. Beaty RM, Edwards JB, Boon K, Siu IM, Conway JE, Riggins GJ (2007) PLXDC1 (TEM 7) is identified in the genome-wide expression screen of the glioblastoma endothelium. J Neurooncol 81:241–248

17. Duesbery NS, Resau J, Webb CP, Koochekpour S, Koo HM, Leppla SH, Vande Woude GF (2001) Suppression of ras-mediated transformation and inhibition of tumor growth and angiogenesis by anthrax lethal factor, a proteolytic inhibitor of multiple MEK pathways. Proc Natl Acad Sci USA 98:4089–4094
18. Nanda A, Carson-Walter EB, Seaman S, Barber TD, Stampfl J, Singh S, Vogelstein B, Kinzler KW, St Croix B (2004) TEM8 interacts with the cleaved C5 domain of collagen alpha3 (VI). Cancer Res 64:817–820
19. Van Baijnun JR, van der Linden E, Zwaans BM, Ramaekers FC, Mayo KH, Griffioen AW (2006) Gene expression of tumor angiogenesis dissected: specific targeting of colon cancer angiogenic vasculature. Blood 108:2339–2348
20. Madden SL, Cook BP, Nacht M, Weber WD, Callahan MR, Jiang Y, Dufault MR, Zhang X, Zhang W, Walter-Yohrling J, Rouleau C, Akmaev VR, Wang CJ, Cao X, St Martin TB, Roberts BL, Teicher BA, Klinger KW, Stan RV, Lucey B, Carson-Walter EB, Laterra J, Walter KA (2004) Vascular gene expression in nonneoplastic and malignant brain. Am J Pathol 165:601–608
21. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, Porter D, Hu M, Chin L, Richardson A, Schnitt S, Sellers WR, Polyak K (2004) Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell 6:17–32
22. Parker BS, Argani P, Cook BP, Liangfeng H, Chartrand SD, Zhang M, Saha S, Bardelli A, Jiang Y, St Martin TB, Nacht M, Teicher BA, Klinger KW, Sukumar S, Madden SL (2004) Alterations in vascular gene expression in invasive breast carcinoma. Cancer Res 64:8757–7866
23. Fonseca R, Barlogie B, Bataille R, Bastard C, Bergsagel PL, Chesi M, Davies FE, Drach J, Greipp PR, Kirsch IR, Kuehl WM, Hernandez JM, Minvielle S, Pilarski LM, Shaughnessy JD Jr, Stewart AK, Avet-Loiseau H (2004) Genetics and cytogenetics of multiple myeloma: a workshop report. Cancer Res 64:1546–1558
24. Mattioli M, Agnelli L, Fabris S, Baldini L, Morabito F, Bicciato S, Verdelli D, Intini D, Nobili L, Cro L, Pruneri G, Callea V, Stelitano C, Maiolo AT, Lombardi L, Neri A (2005) Gene expression profiling of plasma cell dyscrasias reveals molecular patterns associated with distinct IGH translocations in multiple myeloma. Oncogene 24:2461–2473
25. Agnelli L, Bicciato S, Mattioli M, Fabris S, Intini D, Verdelli D, Baldini L, Morabito F, Callea V, Lombardi L, Neri A (2005) Molecular classification of multiple myeloma: a distinct transcriptional profile characterizes patients expressing CCND1 and negative for 14q32 translocations. J Clin Oncol 23:7296–7306
26. Zhan F, Hardin J, Kordsmeier B, Bumm K, Zheng M, Tian E, Sanderson R, Yang Y, Wilson C, Zangari M, Anaissie E, Morris C, Muwalla F, van Rhee F, Fassas A, Crowley J, Tricot G, Barlogie B, Shaughnessy J Jr (2002) Global gene expression profiling of multiple myeloma, monoclonal gammopathy of undetermined significance, and normal bone marrow plasma cells. Blood 99:1745–1757
27. Zhan F, Tian E, Bumm K, Smith R, Barlogie B, Shaughnessy J Jr (2003) Gene expression profiling of human plasma cell differentiation and classification of multiple myeloma based on similarities to distinct stages of late-stage-B-cell development. Blood 101:1128–1140
28. Davies FE, Dring AM, Li C, Rawstron AC, Shammas MA, O'Connor SM, Fenton JA, Hideshima T, Chauhan D, Tai IT, Robinson E, Auclair D, Rees K, Gonzalez D, Ashcroft AJ, Dasgupta R, Mitsiades C, Mitsiades N, Chen LB, Wong WH, Munshi NC, Morgan GJ, Anderson KC (2003) Insights into the multistep transformation of MGUS to myeloma using microarray expression analysis. Blood 102:4504–4511
29. Magrangeas F, Nasser V, Avet-Loiseau H, Loriod B, Decaux O, Granjeaud S, Bertucci F, Birnbaum D, Nguyen C, Harousseau JL, Bataille R, Houlgatte R, Minvielle S (2003) Gene expression profiling of multiple myeloma reveals molecular portraits in relation to the pathogenesis of the disease. Blood 101:4998–5006
30. Roccaro AM, Sacco A, Thompson B, Leleu X, Azab AK, Azab F, Runnels J, Jia X, Ngo HT, Melhem MR, Lin CP, Ribatti D, Rollins BJ, Witzig TE, Anderson KC, Ghobrial IM (2009) MicroRNAs 15a and 16 regulate tumor proliferation in multiple myeloma. Blood 113:6669–6680
31. Ribatti D, Nico B, Crivellato E, Roccaro AM, Vacca A (2007) The history of the angiogenic switch concept. Leukemia 21:44–52

32. Ribatti D, Vacca A, Dammacco F (1999) The role of the vascular phase in solid tumor growth: a historical review. Neoplasia 1:293–302
33. Vacca A, Ribatti D (2006) Bone marrow angiogenesis in multiple myeloma. Leukemia 20:193–199
34. Ribatti D, Nico B, Vacca A (2006) Importance of the bone marrow microenvironment in inducing the angiogenic response in multiple myeloma. Oncogene 25:4257–4266
35. Ribatti D, Vacca A (2009) The role of monocytes-macrophages in vasculogenesis in multiple myeloma. Leukemia 23:1535–1536
36. Vacca A, Semeraro F, Merchionne F, Coluccia M, Boccarelli A, Scavelli C, Nico B, Gernone A, Battelli F, Tabilio A, Guidolin D, Petrucci MT, Ribatti D, Dammacco F (2003) Endothelial cells in the bone marrow of patients with multiple myeloma. Blood 102:3340–3348
37. Pellegrino A, Ria R, Di Pietro G, Cirulli T, Surico G, Pennisi A, Morabito F, Ribatti D, Vacca A (2005) Bone marrow endothelial cells in multiple myeloma secrete CXC-chemokines that mediate interactions with plasma cells. Br J Haematol 129:248–256
38. Ria R, Piccoli C, Cirulli T, Falzetti F, Mangialardi G, Guidolin D, Tabilio A, Di Renzo N, Guarini A, Ribatti D, Dammacco F, Vacca A (2008) Endothelial diferentiation of hematopoietic stem and progenitor cells from patients with multiple myeloma. Clin Cancer Res 14: 1678–1685
39. Coluccia AM, Cirulli T, Neri T, Mangieri D, Colanardi MC, Gnoni A, Di Renzo N, Dammacco F, Tassone P, Ribatti D, Gambacorti-Passerini C, Vacca A (2008) Validation of PDGFRβ and C-Src tyrosine kinases as tumor/vessel targets in patients with multiple myeloma: preclinical efficacy of the novel, orally available inihibitor dasatinib. Blood 112:1346–1356
40. Hedvat CV, Comenzo RL, Teruya-Feldstein J, Olshen AB, Ely SA, Osman K, Zhang Y, Kalakonda N, Nimer SD (2003) Insights into extranedullary tumour cell growth revealed by expressing profiling of human plasmacytomas and multiple myeloma. Br J Haematol 122:728–744
41. Munshi NC, Hideshima T, Carasco D, Shammas M, Auclair D, Davies F, Mitsiades N, Mitsiades C, Kim RS, Li C, Rajkumar SV, Fonseca R, Bergsagel L, Chauhan D, Anderson KC (2004) Identification of genes modulated in multiple myeloma using genetically identical twin samples. Blood 103:1799–1806
42. Vacca A, Scavelli C, Montefusco V, Di Pietro G, Neri A, Mattioli M, Bicciato S, Nico B, Ribatti D, Dammacco F, Corradini P (2005) Thalidomide downregulates angiogenic genes in bone marrow endothelial cells of patients with active multiple myeloma. J Clin Oncol 23:5534–5546
43. Hose D, Moreaux J, Meissner T, Seckinger A, Goldschmidt H, Benner A, Mahtouk K, Hillengass J, Rème T, De Vos J, Hundemer M, Condomines M, Bertsch U, Rossi JF, Jauch A, Klein B, Möhler T (2009) Induction of angiogenesis by normal and malignant plasma cells. Blood 114:128–143
44. Ria R, Todoerti K, Berardi S, Coluccia AM, De Luisi A, Mattioli M, Ronchetti D, Morabito F, Guarini A, Petrucci MT, Dammacco F, Ribatti D, Neri A, Vacca A (2009) Gene expression profiling of bone marrow endothelial cells in patients with multiple myeloma. Clin Cancer Res 15:5369–5378
45. Mizukami Y, Fujiki K, Duerr EM, Fujiki K, Duerr EM, Gala M, Jo WS, Zhang X, Chung DC (2006) Hypoxic regulation of vascular endothelial growth factor through the induction of phosphatidylinositol3-kinase/Rho/ROCK and c-Myc. J Biol Chem 281:13957–13963
46. Wu MX, Ao Z, Prasad KV, Wu R, Schlossman SF (1998) IEX-1 L, an apoptosis inhibitor involved in NF-kB-mediated cell survival. Science 281:998–1001
47. De Vos J, Thykjaer T, Tarte K, Ensslen M, Raynaud P, Requirand G, Pellet F, Pantesco V, Rème T, Jourdan M, Rossi JF, Ørntoft T, Klein B (2002) Comparison of gene expression profiling between malignant and normal plasma cells with oligonucleotide arrays. Oncogene 21:6848–6857
48. Ren JG, Jie C, Talbot C (2005) How PEDF prevents angiogenesis: a hypothesized pathway. Med Hypothesis 64:74–78
49. Volpert OV, Zaichuk T, Zhow W, Reiher F, Ferguson TA, Stuart PM, Amin M, Bouck NP (2002) Inducer-stimulated Fas targets activated endothelium for destruction by anti-angiogenic thrombospondin-1 and pigment epithelium-derived factor. Nat Med 8:349–357

50. Loflin J, Lopez N, Whanger PD, Kioussi C (2006) Selenoprotein W during development and oxidative stress. J Inorg Biochem 100:1679–1884
51. Wang L, Hoque A, Luo RZ, Yuan J, Lu Z, Nishimoto A, Liu J, Sahin AA, Lippman SM, Bast RC Jr, Yu Y (2003) Loss of the expression of the tumor suppressor gene ARHI is associated with progression of breast cancer. Clin Cancer Res 9:3660–3666
52. Thorpe PE (2004) Vascular targeting agents as cancer therapeutics. Clin Cancer Res 10:415–427
53. Ferrara N (2002) Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications. Semin Oncol 19:10–14
54. Pastorino F, Di Paolo D, Loi M, Becherini P, Caffa I, Zorzoli A, Marimpietri D, Carosio R, Perri P, Montaldo PG, Brignole C, Pagnan G, Ribatti D, Allen TM, Ponzoni M (2009) Recent advances in targeted anti-vasculature therapy: the neuroblastoma model. Curr Drug Targets 10:1021–1027

Chapter 14
Epigenetic Regulation of Myeloma Within Its Bone Marrow Microenvironment

Elke De Bruyne, Ken Maes, Sarah Deleu, Els Van Valckenborgh, Eline Menu, Isabelle Vande Broek, Joanna Fraczek, Leo van Grunsven, Vera Rogiers, Helena Jernberg-Wiklund, and Karin Vanderkerken

Abstract Epigenetic mechanisms play a crucial role in the normal development of the mammalian organism and are essential for maintaining the cell identity and normal functionality. Global changes in the epigenetic landscape associated with aberrant gene expression are a hallmark of cancer. Current knowledge indicates that both epigenetic alterations and genetic aberrations play an important role in the onset and progression of cancer. Recent findings have demonstrated that in cancer extensive reprogramming of all components of the epigenetic machinery (including DNA methylation, histone modifications and miRNA expression) takes place and have furthermore revealed the existence of a dynamic interplay between the different components. However, the exact sequence of events and underlying molecular mechanism contributing to carcinogenesis are only just beginning to be uncovered. Interestingly, the reversal of aberrant epigenetic modifications has emerged as a potential treatment strategy of cancer. Here, we describe the role of the epigenetic alterations in the pathogenesis of cancer focusing on the hematological malignancy multiple myeloma. In addition, recent advances regarding the relationship between histone modifications, chromatin-modifying enzymes, DNA methylation and miRNA expression are discussed.

E. De Bruyne • K. Maes • S. Deleu • E. Van Valckenborgh • E. Menu
I. Vande Broek • K. Vanderkerken (✉)
Department of Hematology and Immunology, Vrije Universiteit Brussel,
Laarbeeklaan 103, B-1090 Brussels, Belgium
e-mail: Karin.Vanderkerken@vub.ac.be

L. van Grunsven
Department of Liver cell Biology, Vrije Universiteit Brussel, Brussels, Belgium

V. Rogiers • J. Fraczek
Department of Toxicology, Vrije Universiteit Brussel, Brussels, Belgium

H. Jernberg-Wiklund
Department of Immunology, Genetics and Pathology, Rudbeck Laboratory,
Uppsala University, Uppsala, Sweden

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_14,

14.1 Introduction

One of the key features of multiple myeloma (MM) is the predominant localization of the tumor cells in the bone marrow (BM). The BM microenvironment plays a pivotal role in the MM pathogenesis providing a sanctuary for the MM cells to survive, proliferate, and evade drug-induced cell death due to the existence of functional, mutual interactions between the MM cells and the BM compartments. In addition, environmentally favorable conditions for tumor growth are created by the MM cells by inducing angiogenesis, stimulating osteolysis, and modulating the immune system [1–3]. The list of growth factors produced in the BM microenvironment is continuously expanding, with interleukin-6 (IL-6), insulin like growth factor-1 (IGF-1), IL-1α, IL-1β, vascular endothelial growth factor (VEGF), and stromal-derived factor 1a (SDF1a) being the most important ones [4]. Based on the enhanced understanding of the pivotal role of the intimate reciprocal relationship, numerous new molecular targets have been identified and novel therapies, including compounds such as thalidomide, bortezomib, and lenalidomide, have been introduced [2, 5]. Although in some cases significant improvement in the overall survival has been achieved, MM still remains an incurable disease. Almost all patients eventually relapse, become resistant to the treatment, and die of therapy-refractory disease [5]. To prevent drug resistance and improve patient outcome, it is important to continue preclinical investigation for the identification of novel targets and for the validation of selective drugs, to be used either as a single agent or in combinatorial regimens.

Multiple genetic aberrations are observed during the MM pathogenesis, such as illegitimate rearrangements of the IgH on 14q32, monosomy 13 and deletions of 13q [6]. In addition, a growing number of studies indicate that epigenetic modifications (i.e., heritable changes to the transcriptome without altering the primary DNA sequence) may play a major role in the pathogenesis of MM [7–9]. In this respect, two of the best documented modifications consist of (1) DNA methylation of cytosine bases within a CpG dinucleotide and (2) post-translational (non)histone modifications (PTMs; e.g., acetylation, methylation, phosphorylation) [10, 11]. Both the interaction between these two epigenetic events and the dynamic interplay between the different PTMs are now broadly acknowledged to have a profound impact on the organization of the chromatin architecture and hence on the strict control of gene transcription [10–12]. The fundamental difference between epigenetic and genetic alterations is the reversible nature of the former, allowing for therapeutic intervention. Consequently, over the last 5 years, a plethora of epigenetic-modulating agents have shown promising results in both preclinical and clinical settings and even more prominent in combination with chemotherapeutic agents [13–16].

In addition to DNA methylation and PTMs, microRNAs (miRNAs) form a third post-transcriptional player of gene regulation and are currently intensively studied. Emerging evidence has shown that the expression of miRNA is deregulated in various cancers including MM [17–20]. The scope of this review is to summarize the recent work on epigenetic regulation of MM within its BM microenvironment, with emphasis on the crosstalk between the three major epigenetic regulatory mechanisms.

14.2 DNA Methylation

DNA methylation is a covalent chemical modification on the carbon-5 position of cytosine preceding guanines (CpG dinucleotides). The transfer of a methyl group from the *S*-adenosyl-L-methionine donor to cytosines is catalyzed by DNA methyltransferases (namely DNMT1, DNMT3a, and DNMT3b) [21]. DNMT1, also known as "the maintenance DNMT," copies existing methylation patterns following DNA replication, whereas de novo methylation is attributed to DNMT3a and DNMT3b. Recent studies, however, have indicated that this clear delineation seems to be an oversimplification, as most DNMTs plausibly possess both the de novo and maintenance function as well in vitro as in vivo [21–23]. The pattern of DNA methylation is established during early development and is heritable [24]. In the mammalian genome, CpG sites are unevenly distributed as most of the CpG dinucleotides are found in CpG-rich regions (CpG islands) located at the 5′ end of the regulatory region of approximately 60 % of the human genes [25, 26]. Methylated CpG islands serve as a binding site for methyl-CpG binding domain (MBD) proteins including MeCP2 and MBD1-4. These MBDs interact further with histone modifying enzymes (as discussed below) and transfer methylated DNA into a compacted chromatin environment that is repressive for transcription [27, 28]. In mammalians, most of the promoter-associated CpGs islands are normally unmethylated, thus permitting gene expression, except for tissue-specific genes or genes involved in processes of X chromosome inactivation and imprinting [29]. The remaining of the CpG dinucleotides form long stretches of repetitive sequences and are heavily methylated in normal cells thus govering genomic instability [7, 11].

Aberrant DNA methylation has been shown to be associated with various cancers and includes both global genomic hypomethylation and locus-specific hypermethylation of CpG islands. Hypomethylation is foremost associated with the long stretches of repetitive sequences and transposable elements which leads to activation of these elements and consequently to genomic instability [7, 11, 30]. Gene-specific hypermethylation frequently occurs in the promoter regions of tumor suppressor, cell cycle control, and DNA repair genes, but also in genes involved in cell signaling and cell–cell communication [28]. Interestingly, several sequences have been identified that correlate with methylation susceptibility [31–34].

Today, a number of synthetic and natural DNMT inhibitors (DNMTi) are available. By far the best described and widely used DNMTi are the cytosine nucleoside analogues with modifications at the carbon-5 position of the ring, e.g., 5′azacytidine (AZA) or 5′-aza-2′-deoxycytidine (decitabine, DAC). These molecules are prodrugs that are phosphorylated and subsequently incorporated into DNA and/or RNA. Once incorporated they form covalent bounds with DNMT, resulting in bulky DNA-protein adducts and inhibition of DNA synthesis. Eventually, this will lead to cell death at high doses and passive demethylation upon replication at low doses. Both DAC and AZA have recently been approved by the US Food and Drug administration as antitumor agents for the treatment of myelodysplastic syndrome [15].

14.3 DNA Methylation and DNA Methyltransferase Inhibitors (DNMTi) in Multiple Myeloma

Over the last 5 years, an increasing list of genes known to be implicated in MM pathogenesis were shown to be hypermethylated in MM including CDKN2B, CDKN2A [35], SHP1 [36], SOCS-1 [37], DAP kinase [35], p16, p15 [36, 37], ARF [37], EGLN3 [38], CDH1 [36, 39], RASD1 [40], BNIP3 [36], RAR beta [36], DCL1 [41], MEG3 [42], ICSBP/IRF8 [43], p53 [44], SFRP1,-2 and -5 [45]. Of note, the growth factor IL-6 has been implicated in the maintenance of p53 hypermethylation in MM by increasing DNMT-1 expression [44]. Recent studies have demonstrated that hypermethylation of DAPK [35], EGLN3 [38], RAR beta [36], DCL1 [41], BNIP3 [46], MEG3 [42], SPARC [46], DCC and TGFbetaR2 [47] appears to be an adverse prognostic factor for survival. Moreover, only very recently it was suggested that TP73, ARF, p15, and p16 methylation are early events in the pathogenesis and development of MM, while SOCS-1 methylation would represent an important step in the progression from MGUS to MM [37]. Also, consistent with observations made for other cancers, genomic instability due to global hypomethylation was demonstrated to be correlated with MM progression [48].

Although it is clear that aberrations in DNA methylation are important in MM pathogenesis, only a limited number of studies using DNMTi have been conducted. DAC has been shown to induce p21- and p38-mediated cell cycle G1 phase and G2/M phase arrest, respectively, in MM [49, 50]. Recently, it was also shown that long-term treatment with dexamethasone leads to hypermethylation of the gene RASD1 which results in resistance against dexamethasone [40]. Combination therapy with DAC was demonstrated to overcome this resistance. For AZA, anti-MM activity could be shown by providing evidence that AZA promoted apoptosis, induce double-stranded DNA breaks and inhibit IL-6 and NF-κB signaling pathways [51, 52]. Moreover, AZA was reported to potentiate the anti-MM potential of arsenic trioxide, bortezomib, and doxorubicin [52, 53]. Currently, based on the results of preclinical studies, clinical trials are underway to evaluate the effects of demethylating agents such as DAC and AZA in the treatment of relapsed and refractory MM.

14.4 Histone Modifications

It is now well recognized that chromatin remodeling is one of the main processes involved in epigenetic regulation. The basic structural unit of chromatin is the nucleosome, consisting of 146 base pairs of DNA wrapped around an octamer of core histones. The N-terminal tails of the histones protrude from the DNA helix and are prone to a variety of covalent, reversible modifications, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and glycosylation (Fig. 14.1) [54, 55]. Only the first three types of modifications have been studied extensively [10].

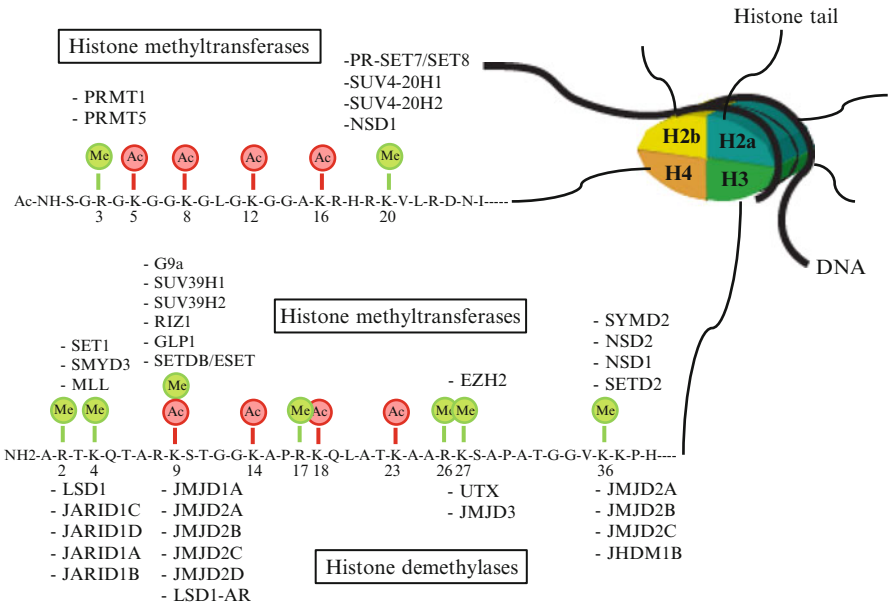


Fig. 14.1 *Schematic overview of histone modifications*. The fundamental repeating unit of chromatin is the nucleosome, consisting of 146 base pairs of DNA wrapped around an octamer of the core histones H2a, H2b, H3, and H4. The N-terminal tails of the histones (especially H3 and H4) protrude from the DNA helix (histone tail domain) and are prone to a variety of covalent, reversible modifications, including acetylation (Ac, *red*), ubiquitination and methylation (Me, *green*) of lysine (K) residues, methylation (Me, *green*) of arginine (R) residues, and phosphorylation of serine (S) and threonine (T) residues. The enzymes responsible for the methylation (histone methyltransferases) and demethylation (histone demethylases) of mammalian H3 and H4 are listed, respectively, above or below their target site. In general, active marks include acetylation, arginine methylation and methylation of lysine 4 of the H3 tail (H3K4) and H3K36. In contrast, repressive marks include H3K9, H3K27, and H4K20 methylation

Although the functional relevance of these individual modifications is often unclear, it is widely acknowledged that the global repertoire of histone modifications constitutes a (epigenetic) code that modulates the accessibility of the chromatin to transcription factors and/or other proteins, thereby controlling gene expression [10, 12].

14.4.1 Histone Acetylation/Deacetylation

Acetylation remains by far the most widely studied histone modification and is governed by opposing actions of a variety of histone deacetylases (HDACs) and histone acetyltransferases (HATs). Acetylation of the lysine residues neutralizes the positive charges of histones, thereby reducing the affinity between histones and DNA and thus leading to a more relaxed, transcriptional active chromatin state. In contrast, hypoacetylation will lead to a dense, transcriptional inactive chromatin. The level of acetylation/deacetylation is controlled by an equilibrium between HATs

and HDACs [56, 57]. Currently, a total of 18 HDAC isoenzymes have been identified in humans and are subdivided into four major classes based on phylogenetic analysis and homology to yeast HDACs. Class I consists of HDAC-1, -2, -3, and −8 while class II is represented by HDAC-4, -5, -6, -7, -9, and 10. HDAC-11 constitutes the single representative of class IV [58, 59]. Class I, II, and IV HDAC enzymes belong to a group of zinc-dependent aminohydrolases owing to the presence of Zn^{2+} in their catalytic center. Class III HDACs, also called sirtuins, constitute a distinct group of HDACs requiring NAD^+ for their catalytic activity. Whereas class I HDACs are rather small proteins with predominantly nuclear localization, class II enzymes are large and capable to translocate between cellular compartments [60]. Among class I, HDAC-1 and -2 seem to regulate most of the changes observed in histone acetylation [61]. Importantly, HDACs do not act autonomously but typically associate with multiprotein complexes as they require DNA-binding proteins to target specific genes [58]. At the same time, other members of the complex act as cofactors, augmenting the enzymatic activity of HDACs [62]. Histones are, however, not the sole target of HDACs/HATs. In fact, a variety of cytoplasmic and nuclear proteins, including transcription factors, DNA repair enzymes, cell-cycle regulators, molecular chaperones as well as structural proteins, are reversibly acetylated and protein modification by acetylation is nearly as abundant as by phosphorylation [63, 64]. Acetylation/deacetylation may modify the stability of the protein, its localization, DNA-binding ability, and its interactions with other proteins [65, 66]. Consequently, a novel nomenclature was proposed for some of the chromatin-modifying enzymes, replacing the historical term of "histone acetyltransferases" (HATs) by the more general one, of "lysine (K)-acetyltransferases" (KATs) [67].

There is increasing evidence that overexpression of HDACs, especially of class I enzymes, correlates with a poor outcome in a wide variety of cancers [59]. In addition, in hematological malignancies, HDACs are recruited by oncogenic fusion proteins to suppress regulatory gene targets [68, 69]. Consequently, a broad range of natural and synthetic HDAC inhibitors (HDACi) have been developed and used as anti-cancer agents both in preclinical and clinical settings [9, 16, 56, 59, 65, 70]. Based on their chemical structure, HDACi can be divided into four major classes, namely short-chain fatty acids (e.g., Valproic acid, Butyrate), hydroxamates (e.g., Suberoylanilide hydroxamic acid, Trichostatin A, LBH589, ITF2357, Tubacin), benzamides (e.g., MS-275 and CI-994), and cyclic tetrapeptides (e.g., depsipeptide, apicidin) [9, 70, 71]. Among these, hydroxamates and cyclic tetrapeptides are considered to be the more potent HDACi. In general, HDAC inhibitors, with the exception of tubacin targeting HDAC6, do not exhibit specificity toward a single specific isoenzyme [9, 71, 72].

14.4.2 Histone Methylation/Demethylation

Histone methylation is governed by specific histone methyltransferases (HMTs) and its mechanism of action is quite different from histone acetylation: (1) histone methylation does not alter the histone tail charge but rather influences its basicity, hydrophobicity,

and the affinity of certain proteins toward DNA [73]; (2) histones can be methylated on both lysines and arginines; (3) histone methylation is often site-specific and can exist in a mono-, di-, or trimethylated state (for arginines only mono- and dimethylation). Depending on the site and degree of methylation, distinct transcriptional readouts will be obtained. For example, while H3K4me2/3 (di-, or trimethylation of lysine 4), H3K36me2/3, and H3K79me generally activate transcription, H3K9me2/3, H3K27me2/3, and H4K20me3 are known to be repressive marks [10, 11, 74].

To date, approximately 50 lysine and arginine HMTs have been identified in humans, with MLL1-4, EZH2, NSD2 (MMSET), SUV39H1/2, G9a and PRMT1, PRMT2, PRMT4, PRMT5, and PRMT7 being only a few of them [10, 75]. At least half of the HMTs have been associated with cancer development and progression [75]. For example, MLL is expressed as an aberrant fusion protein in different types of leukemia [75–77] and EZH2 is overexpressed in a wide variety of tumors [75, 78, 79]. Also, SUV39H1 overexpression was shown to be linked to B-cell lymphoma [80]. Both genetic and epigenetic mechanisms, as well as miRNAs, have been implicated in the deregulation of HMTs in cancer [11, 75, 81]. Some of these HMTs will be discussed below in more detail. To date, only a handful of specific and non-specific HMTs inhibitors have been discovered [74, 82–84]. However, large screening efforts are in progress and will hopefully lead to the identification of additional inhibitors with clinical potential.

For quite some time, histone methylation was considered to be a permanent, irreversible mark [10, 74]. However, recently two classes of bona fide histone demethylases (HDMs), namely, LSD1 and the JmjC domain family, were discovered [85, 86]. The JmjC family is further divided into 5 subfamilies, namely, JHDM1, JHDM2, JMJD2, JMJD3, and JARID1 [87, 88]. LSD1 is only able to turn over mono- and dimethylated lysines, whereas some members of the JmjC family are capable to act on trimethylated lysines as well [74, 89, 90]. Apparently, LSD1 acts in multiprotein complexes with HDACs and MLL [91, 92]. Aberrant expression of HDMs has also been correlated with cancer [74, 93, 94]. Similar to the HMTs inhibitors, the search for HDM inhibitors is still in its infancy and development of selective inhibitors is urgently warranted [74].

Apart from being involved in altering the physical properties of chromatin, a given PTM of a specific histone residue is thought to be determinant to subsequent modifications of that same histone or other ones. Consequently, the context of the encrypted message (namely the histone code) will be changed [10, 95]. This molecular language of chemical marks is deciphered by nuclear proteins such as transcription factors or chromatin modifying enzymes. These are endowed with specific recognition modules with bromo- and chromodomains that recognize acetylated sites or amino acids in methylated form, respectively [96, 97]. Depending on the nature of the recruited protein, a specific action will be initiated. Thus, the factual effect on transcription not only depends on the net electric charge of all modifications on histone tails but also on the type of proteins, i.e., silencers or activators of transcription, recruited in response to displacement of specific moieties. Similar to DNA methylation, aberrant histone modification patterns have been implicated in carcinogenesis. Overall, cancer cells seem to exhibit a global decrease in the levels of acetylation of histone H4 (H4Ac), H4K20me2/3, and H3K9me2 [98–100].

14.5 Histone Deacetylases and HDAC Inhibitors in Multiple Myeloma

While there is no evidence of aberrant HDAC expression or activity in MM, numerous in vitro and preclinical studies have reported potent anti-MM activity of HDACi [8, 9]. Moreover, in MM cells with autocrine IL-6 expression, the chromatin organization of the IL-6 promoter was reported to be different compared to that of non-producing cells [101]. Furthermore, following melphalan treatment, increased acetylation of the oncogenes myc and cyclin D1 was observed in MM patients [102]. In addition, the drug-resistant RPMI8226 cells were demonstrated to exhibit increased chromatin condensation and (over)expression of the ABCB1 gene compared to the sensitive counterpart. ABCB1 has been described in MM as an important resistance mechanism to conventional therapy [103]. Together, these reports support the hypothesis that histone deacetylation plays an important role in MM pathogenesis.

In vitro, several publications showed that a wide range of HDACi induce dose-dependent cell cycle arrest at the G0/G1 phase and this is mostly due to up-regulation of p21 [104–109]. Moreover, HDACi induce apoptosis. However, the mechanism involved seems to be cell specific and HDACi dependent and is still a matter of debate [105, 110, 111]. Although several HDACi activate common apoptotic pathways in the MM cells, significant differences in their mechanism of action exist. For example, TSA, SAHA, LBH589, ITF2357, LAQ824, KD5170, and JNJ-26481585 all activate the intrinsic pathway in MM cells. Of these, SAHA and TSA act in a caspase-independent way, whereas the others are caspase-dependent [104, 106–108, 110, 112]. In addition, HDACi can also up- or down-regulate pro- and anti-apoptotic factors respectively [107, 113–115], induce oxidative stress and DNA damage [112], cause mitochondrial disruption [107, 112], and activate the extrinsic apoptotic pathway [108, 116, 117].

Of importance, HDACi such as VPA, FK288, and IFT2357 affect the viability of both IL-6 dependent and IL-6 independent MM cells lines [106, 109, 114]. Moreover, LBH589, ITF2357, LAQ824, and KD5170 were reported to induce apoptosis as efficiently in the presence or absence of BM stromal cells [105, 106, 110, 112]. This can be explained by the fact that HDACi affect cytokines and proteins that are implicated in MM growth, survival, progression, and immune escape. For example, HDACi were demonstrated to reduce the levels of the growth factors IL-6 and VEGF, secreted by the MM cells and BMSC [105, 106, 108, 109]. Moreover, Mitsiades et al. could demonstrate that SAHA suppresses the expression of IGF-1R, Il-6R and its key signal transducer gp130, TNF-R, syndecan-1 (CD138), and CXCR-4 [118]. Recently, LBH589 and TSA were also demonstrated to increase the expression of tetraspanin CD9 [46, 119]. The latter correlates with non-active MM disease and increases the susceptibility of MM cells to NK cell–mediated cytolysis. In addition to these in vitro experiments, most HDACi were shown to significantly decrease tumor burden and to increase survival in mouse xenograft models [110, 111, 114, 120]. Together, these data clearly indicate that HDACi can overcome the protective signals of the BM microenvironment. This could also be confirmed in the

fully immunocompetent and syngeneic 5TMM models, as the HDACi JNJ-26481585 was reported to not only reduce tumor growth, but also MM-associated bone disease and BM angiogenesis in vivo [104, 121].

As mentioned earlier, HDACs also have numerous non-histone targets. Notably, HDAC6 has emerged as a major deacetylase of α-tubulin and plays a key role in the aggresomal protein degradation system [122], which represents an alternative to the proteasome for degradation of polyubiquitinated misfolded proteins [123]. When the number of misfolded proteins exceeds the proteasomal degradation, the ubiquitinated proteins will form insoluble aggregates termed aggresomes. These will then be transported to the microtubule organizing center (MTOC) for degradation by fusion with lysosomes. This transport requires intact microtubules and association with motor dynein [124, 125]. HDAC6 binds both the polyubiquitinated proteins and dynein, thus facilitating the transport of the aggresomes along the microtubules [122]. Interestingly, HDAC6 was recently identified as a key component of basal autophagy, a process that targets protein aggregates and damaged mitochondria. HDAC6 promotes autophagy by recruiting a cortactin-dependent actin-remodeling machinery by assembly of a F-actin network that stimulates autophagosome-lysosome fusion and substrate degradation [126]. In MM, targeting of HDAC6 with the specific inhibitor tubacin or pan HDACi resulted in accumulation of misfolded proteins, thus leading to apoptosis [105, 127, 128]. In addition, targeting of HDAC6 also impaired the HSP90 chaperone function leading to additional accumulation of aggregates [13, 129]. Consequently, HDACi were shown to significantly enhance the anti-MM activity of proteasome inhibitors and HSP90 inhibitors [105, 127, 130–133]. Furthermore, HDACi are demonstrated to potentiate the anti-MM activity of conventional therapeutic agents such as melphalan, doxorubicin, and dexamethasone [110, 114, 134, 135]. Several clinical trials with HDACi alone or in combination with other anti-MM agents are ongoing and preliminary data look quite encouraging [8, 9]. For a detailed overview of the current ongoing trials, we refer to Chap. 9 of this book.

14.6 MicroRNAs

miRNAs are endogenous, non-coding small (~22 nucleotides) RNAs regulating gene expression in a sequence-specific manner either through degradation of mRNA or translational repression depending on the complementarity between the miRNAs and targets [136, 137]. In the nucleus, miRNA genes are transcribed by RNA polymerase II, yielding long primary miRNA (pri-miRNA) transcripts. These are subsequently cleaved by the nuclear microprocessor complex to 70-nucleotide hairpins, known as precursor miRNA (pre-miRNA). These pre-miRNAs are transported to the cytoplasm by exportin-5, where they are processed by the endonuclease Dicer into 20–22-nucleotide duplexes of mature miRNA. These duplexes are loaded into the RNA-induced silencing (RISC) complex, where the Argonaute protein Ago2 mediates elimination of one of the miRNA strands. The remaining

strand guides RISC to target mRNA that have miRNA complementary sites in the 3′-untranslated region (3′-UTR). RISC then suppresses translation, cleaves or degrades the mRNA depending on the level of complementarity [138, 139]. It is estimated that approximately a 1,000 miRNA species exist in humans. miRNAs are predicted to negatively regulate up to one third of all protein-coding genes, thereby regulating several important biological processes including proliferation, differentiation, and cell death [138, 140–142]. Consequently, miRNA deregulation was extensively reported to correlate with various human cancers and was thought to be involved in the initiation and progression of human cancers [143–145]. Surprisingly, expression profiling of miRNAs was shown to be a more accurate method of classifying cancer subtypes than using expression profiles of protein-coding genes [146, 147]. In addition, large-scale studies demonstrated that miRNA expression signatures are associated with prognosis and response to treatment, underlining their promise as potential cancer biomarkers [18]. miRNAs have been suggested to function either as oncogenes or as tumor suppressor genes. Calin et al. were the first to show that miR-15a and miR-16 (located at chromosome 13q14.3) are frequently down-regulated in patients with B-cell chronic lymphocytic leukemia (B-CLL) and negatively regulate the anti-apoptotic protein Bcl-2, thus acting as tumor suppressors [148]. Other miRNAs that function as tumor suppressors are let-7 and the miR-34 family. miRNAs with a well-established role as oncogenes include the miR-17-92 cluster, miR155 and miR-21 [20, 149]. Over the past few years, an expanding list of miRNAs were suggested to regulate cell proliferation, apoptosis, replicative potential, angiogenesis, immune responses, genomic instability, tumor invasion, and metastasis in/of cancer cells [149]. Consequently, delivery of miRNAs or "antagomirs" that antagonize miRNA function may be an attractive new cancer-treatment modality. However, some caution seems necessary as in most cases an established causal relationship between differential miRNA expression and the malignant phenotype is still lacking. Continued research into miRNA function is therefore warranted in order to advance our understanding of the mechanisms leading to tumorigenesis. Nevertheless, recent findings provided us with some clues. They indicate that transcriptional deregulation, epigenetic alterations, mutations, translocations, DNA copy number abnormalities, and abrogation of global miRNA processing might all cooperate in miRNA deregulation in human cancer [18–20].

14.7 MicroRNAs and Multiple Myeloma

Only a few studies have provided some information about miRNA expression in MM. In 2007, Loffler et al. were the first to show that IL-6 regulates miR-21 transcription in IL6-dependent cell lines through a STAT-3 mechanism and that ectopic miR-21 expression sustained their growth in the absence of IL-6 [150]. One year later, Pichiorri et al. described a MM miRNA signature, which included miR-21, the miR-106b-25 cluster, miR-181a and b, miR-32 and the miR-17-92 cluster and is able to modulate the expression of proteins critical for MM pathogenesis

(e.g., SOCS-1 and p300-CBP-associated factor) [151]. At the same time, miR-15a and miR-16 were reported to be down-regulated in relapsed-refractory MM patients and to regulate proliferation and angiogenesis in MM [152]. Moreover, their anti-MM activity was demonstrated even in the context of the BM microenvironment. According to Corthals et al., there was no correlation between miR-15a and miR-16 expression and chromosome 13 deletions in MM [153]. In chronic lymphocytic leukemia (CLL), however, absence of miR15 and -16 was suggested to be the result of deletion of the DLEU2 gene consistently lost in cases of CLL with a 13q14.3 deletion [154]. Recently, a series of independent studies reported deregulated miRNA expression in primary human MM cells. This seemed to be associated with cytogenetic abnormalities (such as the IgH translocations, deletions and hyperdiploidy) and correlated with gene expression changes characteristic of MM genetic subtypes [155–157]. One of the most interesting findings was the significant upregulation of the miR-1/miR-133a cluster in MM samples with the translocation t(14;16). In addition, it was speculated that the underexpression of miR-196b, miR-135b, miR-320, miR-20a, miR-19b, miR-19a, and miR-15a, found in many MM samples, could contribute to the overexpression of the predicted target CCND2. Of note, it was suggested that deregulation of a CYCLIN D gene is a unifying oncogenic event in MM [6]. Only very recently, Zhou et al. reported that there is a global elevation of miRNAs in high-risk disease defined by the validated 70-gene risk score and proliferation index, which is partially due to overexpression of EIF2C2/AGO2 [158]. This gene is a component of the 70-gene mRNA risk model, its overexpression in MM is driven by DNA copy number gains, and it seems to be the master regulator of maturation and function of miRNAs [158–161]. Based on these data, the authors suggested that the observed genome-wide elevated expression of miRNAs in high-risk MM may be secondary to deregulation of AGO2 and enzyme complexes that regulate miRNA maturation and function. So far only one study has evaluated the functional role of the identified miRNAs in the MM pathogenesis in vivo [151]. Xenograft studies using human MM cell lines treated with miR-19a and b, and miR-181a and b antagonists resulted in significant suppression of tumor growth. Consequently, the functional characterization of the miRNAs described in MM is of outmost importance.

14.8 Interplay Between Histone Modifications, DNA Methylation, and miRNAs and Their Potential as Targets for Intervention in Multiple Myeloma

A growing body of evidence has pointed to the interdependent relationship between histone modifications and DNA methylation [10–12]. In fact, in cancer cells, heavily methylated promoter regions of tumor suppressor genes are often associated with a loss of histone acetylation and enrichment of histone H3K9me2/3 [11, 99]. However, the exact sequence of events and underlying molecular mechanisms are yet unknown. Early studies indicated that the link between histone modifications

and DNA methylation could be dependent on MBD proteins. The latter recruit chromatin-modifying complexes associated with HDAC and HMT activities to sites of de novo DNA methylation, thereby inducing chromatin structural changes [27, 28]. Thus, initially DNA methylation was thought to unidirectionally affect chromatin structure. However, mutations in SUV39H1/2 were shown to reduce DNA methylation, indicating that H3K9me3 might act upstream of DNA methylation [162, 163]. In addition, G9a has also been demonstrated to play an important role in de novo DNA methylation of embryonic and germline genes during normal development [164, 165]. Interestingly, the G9a-mediated DNA methylation does not seem to depend on its catalytic activity (H3K9me2) but rather on its capacity to recognize methyl-lysines and/or to recruit DNMTs [164, 166]. Based on this knowledge, H3K9me2 and DNA methylation have currently been proposed to form a reinforcing silencing loop leading to stable silencing [167–169]. Consequently, G9a was proposed to be one of the key enzymes in the aberrant silencing in cancer. Indeed, G9a is enriched at the promoters of aberrantly methylated genes [169]. Recent evidence also indicates that inhibition of G9a is sufficient to reactivate silenced metastasis suppressor genes and this effect is further enhanced by concurrent inhibition of DNMT1 [170].

In contrast, H3K4me2/3 was suggested to protect promoters from de novo DNA methylation in somatic cells by preventing contact between the nucleosomes and DNTM3L [171–173]. In actively transcribed genes, H3K4me2/3 marks most CpG islands as a result of the recruitment of specific H3K4 HMTs by polymerase II [172]. Consequently, de novo DNA methylation seems to be closely associated with the presence of HDMs [174]. Interestingly, in embryonic stem (ES) cells, the active mark H3K4me2/3 and repressive mark H3K27me3 coexist on development-related genes and this bivalent modification status is believed to be necessary to sustain the undifferentiated state. During ES cell differentiation, the bivalent modifications resolve into either H3K4me2/3 associated with active transcription or H3K27me3 associated with transcriptional repression [175, 176]. Trimethylation of histone H3 at K27 is the central event linking the Polycomb group (PcG) proteins to this transcriptional repression and gene silencing [177]. The PcG proteins function in large multimeric complexes, of which the most well characterized are Polycomb repressive complexes PRC1 and PRC2 [178, 179]. The PRC2 is composed of SUZ12, EED, YY1, and the catalytic subunit EZH2. The HMT activity of EZH2 catalyzes H3K27-trimethylation, serving as a signal to recruit the PRC1. This complex consists of HPC, BMI1, RING1, and HPH and will induce transcriptional silencing by preventing initiation of transcription by RNA polymerase II [180–183]. In line with the assigned role for PcG as preservers of gene silencing required for self-renewal in cells with embryonic origin, it is not surprising that components of the PcG proteins recently also gained wide interest as prominent players in carcinogenesis [184, 185]. EZH2 has previously been reported to be frequently overexpressed in a large number of tumors, in some cases correlating with poor prognosis [79, 186–188]. Consistent with these findings, it was recently found that overexpression of the PRC2 components EZH2, SUZ12, and EED significantly correlates with establishment and progression of MM [189]. EZH2 was associated

with cell proliferation and its expression was induced upon S phase entry and G2/M transition. Several reports also indicate that tumors are dependent on EZH2 for proliferation [81, 188, 190–192]. In MM, EZH2 expression was shown to be induced by IL-6 in growth factor-dependent cell lines, while being constitutively expressed in IL-6-independent cell lines [188]. Also, PcG proteins were suggested to induce tumorigenesis via direct transcriptional repression of key tumor suppressor genes like, $p16^{INK4A}$, $p14^{ARF}$, and E-cadherin [75]. Interestingly, this EZH2-mediated cell cycle progression promoted by gene repression also seems to involve deacetylation by HDAC1 [193]. Consequently, treatment of leukemia cells with the HDACi LBH589 was demonstrated to deplete protein levels of EZH2, EED, and SUZ12, with concomitant reduction of H3K27me2/3 levels and loss of clonogenic survival [194]. Consistent with the interaction of DNMT1, DNMT3A, and 3B with the PRC2 complex, PcG-mediated silencing may also predispose target genes to DNA methylation [195–199]. Furthermore, PcG proteins seem to be necessary for the maintenance of some CpG island methylation patterns in both normal and cancer cells [200]. Recently, however, PcG-mediated H3K27me3 has been suggested to be a solid silencing machinery independent of DNA methylation in human cancers [11, 168]. Apparently at least three different forms of silencing ranging from a flexible and plastic repressor-based mechanism to a highly stable inactivation can be operative in cancer cells (Fig. 14.2) (1) genes can be de novo repressed by PcG-mediated H3K27me3 without DNA methylation [201]; (2) genes can lose their initial H3K27me3 marks after acquiring de novo DNA methylation and H3K9 methylation [168, 201]; and (3) H3K27me3 and DNA methylation may co-exist on certain genes [202]. In this case, the PcG-mediated H3K27me3 is the dominant silencing mechanism [168]. Of note, in prostate cancers, the majority of the genes enriched with the H3K27me3 mark seem to lack a CpG island in the promoter region and thus fall into the first class [168]. Recent data from Kalushkova et al. now show that genes bound by the PcG in cells of embryonic origin overlap with the MM gene profile of underexpressed genes, as compared to normal plasma cells [189]. Underlining its clinical importance, underexpression of genes within the defined profile was more pronounced in the advanced stages (ISS stage III compared to stage II and I) of MM progression. These findings have implications in the understanding of how MM cells retain their "stemness," e.g., the capacity for self-renewal and drug resistance. Unraveling the biological significance of reactivating a silenced gene signature in MM will most certainly have large therapeutic implications. Strategies to chemically revert the H3K27me3-mediated gene silencing by targeting of the PRC2 complex in vitro and in vivo imply that the Polycomb-target gene profile could be highly relevant for pharmacological treatment of MM [189]. The aberrant activity of the HMT WHSC1 (MMSET) or loss of the HDM KDM6A (UTX null mutation) [203, 204] suggests the existence of several possible scenarios to preserve the gene silencing by PcG in a permanent mode in MM. MMSET is involved in the recurrent immunoglobulin translocation t(4;14), associated with a significant poor prognosis [6, 205]. Interestingly, the *UTX* null mutation and t(4;14) translocation were suggested to be mutually exclusive [204]. Taken together, the complex relationship between the histone code and DNA methylation

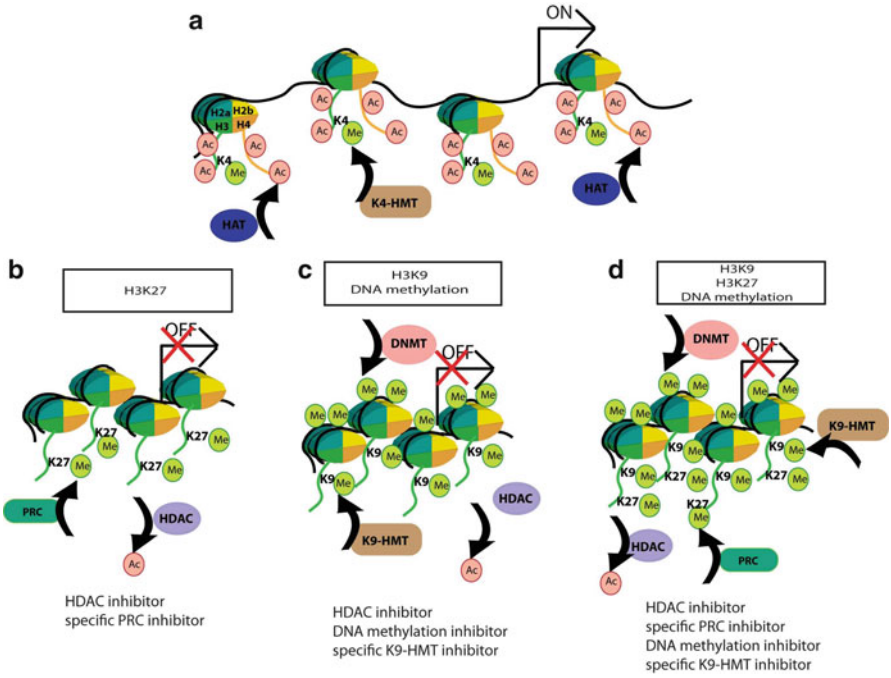


Fig. 14.2 *Overview of the epigenetic gene-silencing mechanisms in cancer.* (**a**) Active genes show an open chromatin state displaying an unmethylated promoter together with enrichment of acetylation of the lysine residues on histone tail H3 and H4 (*red circles*) and trimethylation of H3K4 (H3K4me3) (*green circles*). The combination of these epigenetic modifications allows for transcription if the appropriate transcription factors and enhancers are present. In cancer cells, three different forms of gene silencing, ranging from a flexible and plastic repressor-based mechanism to a highly stable silencing, have been demonstrated. (**b**) Promoters lacking CpG islands are repressed through polycomb group (PcG)-mediated trimethylation of H3K27 (H3K27me3) accompanied by the loss of histones acetylation and removal of H3K4me3. This form of silencing is rather flexible and genes could potentially be fully re-expressed by treatment of the cells with a histone deacetylase inhibitor (HDACi) and/or a specific histone methyltransferase inhibitor (HMTi; e.g., a specific PRC inhibitor). (**c**) Promoters with CpG islands are repressed through DNA methylation (*green circles*) by DNA methyltransferases (DNMTs). Here, DNA methylation is often accompanied by H3K9me2 forming a reinforcing loop leading to highly stable silencing. In addition, DNA-mediated silenced promoters display a loss of H3K4me3 and histone acetylation. Full re-expression of these genes can potentially be achieved by the combination of a DNMT inhibitor (DNMTi) and HDACi. (**d**) However, on some CpG islands rich promoters, the repressive mark H3K27me3 and DNA methylation co-exist. Consequently, these promoters display DNA methylation accompanied with enrichment in the repressive marks H3K27me3 and H3K9me2 and loss of histone acetylation and H3K4me3. In these cases, PcG-mediated H3K27me3 appears to be the dominant silencing mechanism. Full re-expression of these genes could possibly be achieved by the combination of HDACi, DNMTi and/or specific HMTi

provides a rationale for a multifaceted strategy in which multiple components of the epigenetic machinery may be targeted alone or simultaneously in order to achieve complete epigenetic reprogramming of cancer cells. As an example of this, HDACi were shown to affect DNMT activity accompanied by global and gene-specific demethylation or depletion of the repressive chromatin marks [206–210]. Moreover, it has

been reported that treatment with DAC only transiently induces demethylation in the silenced tumor suppressor genes [211]. This phenomenon might be partially explained by the epigenetic memory retained in the chromatin. Consequently, it seems plausible that combining DNTMi and HMTi/HDACi might overcome this problem. Indeed, in vitro, this strategy has proven successfully with the combination of DNMTi and HDACi showing robust re-expression of silenced genes accompanied by an enhanced anticancer response [13, 212, 213]. In leukemia, in vitro synergistic anti-tumor activity of HDACi (VPA, JNJ-26481585 and LBH589) and DAC was demonstrated [206, 214, 215]. So far, no combinatorial therapy targeting DNMTs and HDACs were conducted in MM. However, Heller et al. have reported on the synergy of the DAC and TSA in the re-expression of genes silenced by DNA methylation in MM [46]. In addition, DAC was demonstrated to enhance the LBH589-mediated de-repression of Bim and CD9 in MM cells [113, 119]. In the case of Bim, microenvironmentally produced growth factors, i.e., IGF-1, were recently demonstrated to simultaneously increase H3K9me2 and decrease H3K4me3 and H3K9Ac marks at the Bim and FoxO3a promoter, resulting in silencing of Bim [113]. Interestingly, combinatorial treatment with DAC and the HDACi LBH589 was demonstrated to result in a drastic and significant reduction of the H3K9me3 marks accompanied by an increase in H3K9Ac. It therefore seems plausible that a combinatorial approach targeting several layers of gene silencing favoring tumor growth holds promise also in MM.

An additional group of critical regulators of the classical epigenetic machinery consists of epi-miRNAs, a subset of miRNAs (Fig. 14.3). Recent data show that Dicer$^{-/-}$ ES cells exhibit considerable loss of DNA methylation [216, 217]. Indeed, the number of studies demonstrating that DNMT-1, DNMT-3a, and DNMT-3b are miRNA targets is rapidly growing [218]. For example, the miR-29 family has been shown to directly target DNMT3a and DNMT3b and indirectly DNMT1 [219, 220]. DNMT3b is also under the control of the miR-148 family [221] and indirectly DNMT3a and DNMT3b are controlled by the miR-290 cluster that directly targets RBL2 [216]. Moreover, in human hepatocytes, miR-1 was recently found to target HDAC4, resulting in the onset of hepatocellular cell cycling [222]. HDAC4, on its turn, is targeted by miR-140, while miR-449a targets HDAC1 [223, 224]. In addition, a decrease in miR-101 expression due to genomic loss concomitant with increased expression of EZH2 expression was observed in progressed prostate cancer and bladder transitional cell carcinoma [81, 225]. Likewise, in B-cell lymphoma, miR-26a expression was decreased and inversely correlated with EZH2 levels [226]. So far, there are no reports on epi-miRNAs in MM.

Conversely, DNA methylation and histone modifications have also been suggested to affect miRNA expression in a number of (cancer) cell types [19, 20, 227, 228]. Saito et al. were one of the first to show, by extensive profiling of miRNAs, that 17 of the 313 human miRNAs examined were upregulated after combinatorial treatment with DAC and the HDACi PBA in T24 human bladder cancer cells [227]. Among the re-expressed miRNAs was miR-127 targeting the proto-oncogene BCL6. This tumor suppressor miRNA was demonstrated to be embedded in a CpG island and to be epigenetically silenced by both promoter hypermethylation and histone

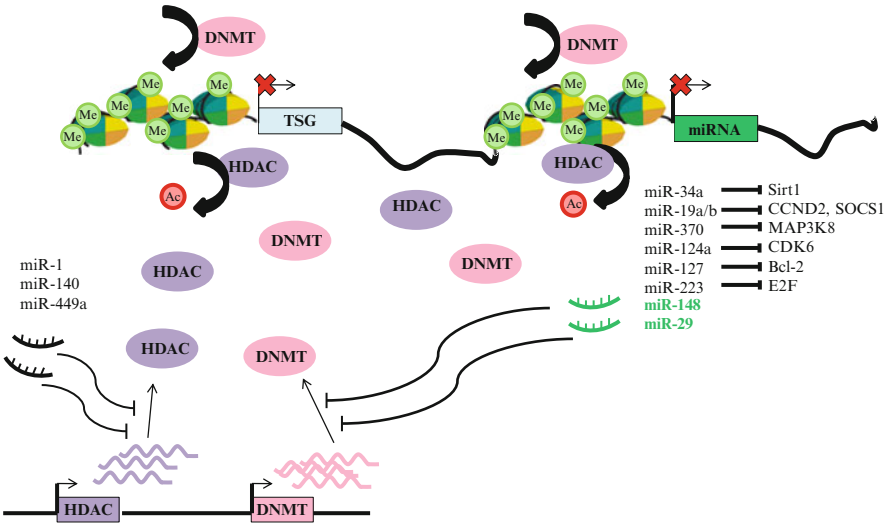


Fig. 14.3 *microRNAs (miRNAs) play a complex role in the regulation of the epigenetic landscape*. miRNAs are ~22 nucleotides small non-coding RNAs which regulate gene expression either through mRNA degradation or translational repression depending on the complementarity between the miRNAs and target genes. miRNA expression patterns are often found deregulated in cancer. miRNA can act as oncogenes or tumor suppressors by inhibiting the expression of cancer-related target genes. Interestingly, a reciprocal interaction exists between miRNA, one the one hand, and the "classical" epigenetic mechanisms (namely histone modifications and DNA methylation), on the other hand. At least two families of miRNAs have been reported to affect expression of the DNMTs. The miR-148 family has been shown to regulate expression of DNMT3b, whereas the miR-29 family has been shown to target DNMT3a, DNMT3b and indirectly DNMT1. In addition, miRNAs have also been shown to regulate expression of HDACs. HDAC4 has been reported to be the target of miR-140 and miR-1, whereas HDAC1 is targeted by miR-449a. The subset of miRNAs that target the classical epigenetic machinery are termed epi-miRNAs. Of note, in cancer cells, the miR-29 and -148 promoters have been reported to be hypermethylated in association with deacetylation, thus leading to elevated levels of DNMTs. In addition, epigenetic silencing of some of the miRNAs known to target critical factors involved in oncogenesis has been documented in cancer

modifications in cancer. The regulation of miRNA expression by DNA methylation was further confirmed by creating double knockout (DKO) for DNMT1 and DNMT3b in the colorectal cancer cell line HTC-116. Eighteen of the 320 miRNAs examined were upregulated in the DKO cells. One of the main targets was miR124a, of which the promoter is densely methylated in cancer cells. miR124a targets the oncogene CDK6 and has an impact on the phosphorylation status of the downstream protein Rb [229]. Recently, promoter hypermethylation of miR-124a was demonstrated to be an independent prognostic factor in acute lymphoblastic leukemia [230]. Moreover, the epi-miRNA families miR-29 and -148 were demonstrated to be hypermethylated in lung cancers [219, 221, 231]. HDACi have also been shown to regulate miRNA expression on their own, e.g., in breast cancer, the HDACi LAQ824 significantly altered miRNA expression levels [228]. Interestingly, it also seems that

transcription factors can recruit epigenetic effectors to the miRNA promoter regions. This was, e.g., shown in acute myeloid leukemia, where the AML1/ETO fusion oncoprotein was reported to bind to the promoter of miR-223 and subsequently recruiting MBP, HDAC1, and DNMTs [232]. In addition, in cholangiocarcinoma, IL-6 increased DNMT1 concomitant with silencing of the promoter of miRNA-370 and re-expressing of the target gene MAP3K8 [233]. It therefore can be speculated that growth factors might be involved in miRNA-modulated tumor development and/or progression. Some reports further indicate that silencing of intronic miRNAs (miRNAs located in an intron of the host gene) might simply be the result of epigenetic changes of the host gene [234, 235].

Very recently, the HDACi IFT2357 was demonstrated to significantly down-regulate miR-19a and -19b expression in several human MM cell lines [236]. In addition, the promoter of miR-34a was found to be methylated in 5.5 % and 18.8 % of primary MM and non-Hodgkin lymphoma respectively at the time of diagnosis. MiR-34a is the transcriptional target of p53 and is implicated in carcinogenesis [237].

Taking all these observations into account, it can be postulated that miRNAs, in concert with the "classical epigenetic mechanisms," could provide a major regulatory platform that drives gene expression. Consequently, it can be anticipated that miRNAs, in combination with HDACi and/or DNMTi represent innovative targets to epigenetically reprogram cancer cells.

14.9 Concluding Remarks

Recent advances in the rapidly evolving field of cancer epigenetics have led to the concept that both the specific organization of the chromatin structure and the DNA methylation process contribute significantly to the pathogenesis of both solid and hematological cancers including MM. Consequently, in an attempt to epigenetically reprogram the cancer cells, a plethora of epigenetic modulating agents such as HDACi and DNMTi have been developed. Of these, a number of compounds demonstrate efficacy as single agents in in vitro and preclinical settings. Moreover, results from ongoing clinical trials are encouraging, especially when given in combination with current therapeutic regimens. However, the molecular mechanisms underlying the origin of cancer-specific DNA methylation, histone modifications, and miRNA expression are still largely unknown and research in this field is just starting. In-depth understanding of the intimate and reciprocal relationship between all the components of the epigenetic machinery and especially of the precise sequential timing of all molecular events in cancer development will not only enhance our comprehension of the different biological mechanisms that drive cancer development and progression, but will also provide new molecular targets (e.g., newly identified HMT and HDM). In addition, the enhanced knowledge will enable the design of better combinatory treatment strategies and the identification of molecular markers for early detection of cancer, prediction of prognosis, and prediction of treatment outcomes.

Acknowledgments The work in the laboratories was financially supported by the International Myeloma Foundation, Multiple Myeloma Research Foundation, Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO-Vl), Belgische Federatie tegen Kanker, Vlaamse Liga tegen Kanker – Stichting Emmanuel Van der Schueren, European Stem cell network (EUFP6 MSCNET and EUFP7 Overmyr), Onderzoeksraad Vrije Universiteit Brussel (GOA48 and GOA78), Swedish Cancer Society and Swedish Research Council. E. De Bruyne, I. Vande Broek, E. Menu, and E. Van Valckenborgh are postdoctoral fellows of FWO-Vl.

References

1. Podar K, Chauhan D, Anderson KC (2009) Bone marrow microenvironment and the identification of new targets for myeloma therapy. Leukemia 23:10–24
2. Podar K, Richardson PG, Hideshima T, Chauhan D, Anderson KC (2007) The malignant clone and the bone-marrow environment. Best Pract Res Clin Haematol 20:597–612
3. Yasui H, Hideshima T, Richardson PG, Anderson KC (2006) Novel therapeutic strategies targeting growth factor signalling cascades in multiple myeloma. Br J Haematol 132:385–397
4. Hideshima T, Podar K, Chauhan D, Anderson KC (2005) Cytokines and signal transduction. Best Pract Res Clin Haematol 18:509–524
5. Raab MS, Podar K, Breitkreutz I, Richardson PG, Anderson KC (2009) Multiple myeloma. Lancet 374:324–339
6. Chng WJ, Glebov O, Bergsagel PL, Kuehl WM (2007) Genetic events in the pathogenesis of multiple myeloma. Best Pract Res Clin Haematol 20:571–596
7. Galm O, Herman JG, Baylin SB (2006) The fundamental role of epigenetics in hematopoietic malignancies. Blood Rev 20:1–13
8. Smith EM, Boyd K, Davies FE (2009) The potential role of epigenetic therapy in multiple myeloma. BrJ Haematol 148(5):702–713
9. Deleu S (2009) Histone deacetylase inhibitors in multiple myeloma. Hematol Rev 1:46–56
10. Margueron R, Trojer P, Reinberg D (2005) The key to development: interpreting the histone code? Curr Opin Genet Dev 15:163–176
11. McCabe MT, Brandes JC, Vertino PM (2009) Cancer DNA methylation: molecular mechanisms and clinical implications. Clin Cancer Res 15:3927–3937
12. Fuks F (2005) DNA methylation and histone modifications: teaming up to silence genes. Curr Opin Genet Dev 15:490–495
13. Bots M, Johnstone RW (2009) Rational combinations using HDAC inhibitors. Clin Cancer Res 15:3970–3977
14. Gilbert J, Gore SD, Herman JG, Carducci MA (2004) The clinical application of targeting cancer through histone acetylation and hypomethylation. Clin Cancer Res 10:4589–4596
15. Issa JP, Kantarjian HM (2009) Targeting DNA methylation. Clin Cancer Res 15:3938–3946
16. Prince HM, Bishton MJ, Harrison SJ (2009) Clinical studies of histone deacetylase inhibitors. Clin Cancer Res 15:3958–3969
17. Gutierrez NC, Sarasquete ME, Misiewicz-Krzeminska I et al (2010) Deregulation of microRNA expression in the different genetic subtypes of multiple myeloma and correlation with gene expression profiling. Leukemia 24:629–637
18. Deng S, Calin GA, Croce CM, Coukos G, Zhang L (2008) Mechanisms of microRNA deregulation in human cancer. Cell Cycle 7:2643–2646
19. Valeri N, Vannini I, Fanini F, Calore F, Adair B, Fabbri M (2009) Epigenetics, miRNAs, and human cancer: a new chapter in human gene regulation. Mamm Genome 20:573–580
20. Yang N, Coukos G, Zhang L (2008) MicroRNA epigenetic alterations in human cancer: one step forward in diagnosis and treatment. Int J Cancer 122:963–968
21. Bestor TH (2000) The DNA methyltransferases of mammals. Hum Mol Genet 9:2395–2402

22. Kim GD, Ni J, Kelesoglu N, Roberts RJ, Pradhan S (2002) Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases. EMBO J 21:4183–4195
23. Liang G, Chan MF, Tomigahara Y et al (2002) Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. Mol Cell Biol 22:480–491
24. Turker MS (1999) The establishment and maintenance of DNA methylation patterns in mouse somatic cells. Semin Cancer Biol 9:329–337
25. Antequera F, Bird A (1993) Number of CpG islands and genes in human and mouse. Proc Natl Acad Sci USA 90:11995–11999
26. Antequera F, Bird A (1993) CPG islands. EXS 64:169–185
27. Bird AP, Wolffe AP (1999) Methylation-induced repression–belts, braces, and chromatin. Cell 99:451–454
28. Gopalakrishnan S, Van Emburgh BO, Robertson KD (2008) DNA methylation in development and human disease. Mutat Res 647:30–38
29. Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 3:662–673
30. Wilson AS, Power BE, Molloy PL (2007) DNA hypomethylation and human diseases. Biochim Biophys Acta 1775:138–162
31. Feltus FA, Lee EK, Costello JF, Plass C, Vertino PM (2003) Predicting aberrant CpG island methylation. Proc Natl Acad Sci USA 100:12253–12258
32. Feltus FA, Lee EK, Costello JF, Plass C, Vertino PM (2006) DNA motifs associated with aberrant CpG island methylation. Genomics 87:572–579
33. Handa V, Jeltsch A (2005) Profound flanking sequence preference of Dnmt3a and Dnmt3b mammalian DNA methyltransferases shape the human epigenome. J Mol Biol 348:1103–1112
34. Keshet I, Schlesinger Y, Farkash S et al (2006) Evidence for an instructive mechanism of de novo methylation in cancer cells. Nat Genet 38:149–153
35. Chim CS, Kwong YL, Liang R (2008) Gene hypermethylation in multiple myeloma: lessons from a cancer pathway approach. Clin Lymphoma Myeloma 8:331–339
36. Braggio E, Maiolino A, Gouveia ME et al (2010) Methylation status of nine tumor suppressor genes in multiple myeloma. Int J Hematol 91:87–96
37. Stanganelli C, Arbelbide J, Fantl DB, Corrado C, Slavutsky I (2010) DNA methylation analysis of tumor suppressor genes in monoclonal gammopathy of undetermined significance. Ann Hematol 89:191–199
38. Hatzimichael E, Dasoula A, Shah R et al (2010) The prolyl-hydroxylase EGLN3 and not EGLN1 is inactivated by methylation in plasma cell neoplasia. Eur J Haematol 84:47–51
39. Seidl S, Ackermann J, Kaufmann H et al (2004) DNA-methylation analysis identifies the E-cadherin gene as a potential marker of disease progression in patients with monoclonal gammopathies. Cancer 100:2598–2606
40. Nojima M, Maruyama R, Yasui H et al (2009) Genomic screening for genes silenced by DNA methylation revealed an association between RASD1 inactivation and dexamethasone resistance in multiple myeloma. Clin Cancer Res 15:4356–4364
41. Ullmannova-Benson V, Guan M, Zhou X et al (2009) DLC1 tumor suppressor gene inhibits migration and invasion of multiple myeloma cells through RhoA GTPase pathway. Leukemia 23:383–390
42. Benetatos L, Hatzimichael E, Dasoula A et al (2010) CpG methylation analysis of the MEG3 and SNRPN imprinted genes in acute myeloid leukemia and myelodysplastic syndromes. Leuk Res 34:148–153
43. Tshuikina M, Jernberg-Wiklund H, Nilsson K, Oberg F (2008) Epigenetic silencing of the interferon regulatory factor ICSBP/IRF8 in human multiple myeloma. Exp Hematol 36:1673–1681
44. Hodge DR, Peng B, Cherry JC et al (2005) Interleukin 6 supports the maintenance of p53 tumor suppressor gene promoter methylation. Cancer Res 65:4673–4682
45. Jost E, Schmid J, Wilop S et al (2008) Epigenetic inactivation of secreted Frizzled-related proteins in acute myeloid leukaemia. Br J Haematol 142:745–753

46. Heller G, Schmidt WM, Ziegler B et al (2008) Genome-wide transcriptional response to 5-aza-2′-deoxycytidine and trichostatin a in multiple myeloma cells. Cancer Res 68:44–54
47. de Carvalho F, Colleoni GW, Almeida MS, Carvalho AL, Vettore AL (2009) TGFbetaR2 aberrant methylation is a potential prognostic marker and therapeutic target in multiple myeloma. Int J Cancer 125:1985–1991
48. Bollati V, Fabris S, Pegoraro V et al (2009) Differential repetitive DNA methylation in multiple myeloma molecular subgroups. Carcinogenesis 30:1330–1335
49. Lavelle D, De Simone J, Hankewych M, Kousnetzova T, Chen YH (2003) Decitabine induces cell cycle arrest at the G1 phase via p21(WAF1) and the G2/M phase via the p38 MAP kinase pathway. Leuk Res 27:999–1007
50. Zhu WG, Hileman T, Ke Y et al (2004) 5-aza-2′-deoxycytidine activates the p53/p21Waf1/Cip1 pathway to inhibit cell proliferation. J Biol Chem 279:15161–15166
51. Khong T, Sharkey J, Spencer A (2008) The effect of azacitidine on interleukin-6 signaling and nuclear factor-kappaB activation and its in vitro and in vivo activity against multiple myeloma. Haematologica 93:860–869
52. Kiziltepe T, Hideshima T, Catley L et al (2007) 5-Azacytidine, a DNA methyltransferase inhibitor, induces ATR-mediated DNA double-strand break responses, apoptosis, and synergistic cytotoxicity with doxorubicin and bortezomib against multiple myeloma cells. Mol Cancer Ther 6:1718–1727
53. Chen G, Wang Y, Huang H et al (2009) Combination of DNA methylation inhibitor 5-azacytidine and arsenic trioxide has synergistic activity in myeloma. Eur J Haematol 82:176–183
54. Davie JR (1998) Covalent modifications of histones: expression from chromatin templates. Curr Opin Genet Dev 8:173–178
55. Nightingale KP, O'Neill LP, Turner BM (2006) Histone modifications: signalling receptors and potential elements of a heritable epigenetic code. Curr Opin Genet Dev 16:125–136
56. Kim TY, Bang YJ, Robertson KD (2006) Histone deacetylase inhibitors for cancer therapy. Epigenetics 1:14–23
57. Yang XJ, Seto E (2008) Lysine acetylation: codified crosstalk with other posttranslational modifications. Mol Cell 31:449–461
58. Yang XJ, Seto E (2007) HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. Oncogene 26:5310–5318
59. Schrump DS (2009) Cytotoxicity mediated by histone deacetylase inhibitors in cancer cells: mechanisms and potential clinical implications. Clin Cancer Res 15:3947–3957
60. Gregoretti IV, Lee YM, Goodson HV (2004) Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. J Mol Biol 338:17–31
61. Suzuki J, Chen YY, Scott GK et al (2009) Protein acetylation and histone deacetylase expression associated with malignant breast cancer progression. Clin Cancer Res 15:3163–3171
62. Walkinshaw DR, Tahmasebi S, Bertos NR, Yang XJ (2008) Histone deacetylases as transducers and targets of nuclear signaling. J Cell Biochem 104:1541–1552
63. Glozak MA, Sengupta N, Zhang X, Seto E (2005) Acetylation and deacetylation of non-histone proteins. Gene 363:15–23
64. Choudhary C, Kumar C, Gnad F et al (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science 325:834–840
65. Minucci S, Pelicci PG (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer 6:38–51
66. Xu WS, Parmigiani RB, Marks PA (2007) Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene 26:5541–5552
67. Allis CD, Berger SL, Cote J et al (2007) New nomenclature for chromatin-modifying enzymes. Cell 131:633–636
68. Lin RJ, Sternsdorf T, Tini M, Evans RM (2001) Transcriptional regulation in acute promyelocytic leukemia. Oncogene 20:7204–7215
69. Wang J, Hoshino T, Redner RL, Kajigaya S, Liu JM (1998) ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. Proc Natl Acad Sci USA 95:10860–10865

70. Vanhaecke T, Papeleu P, Elaut G, Rogiers V (2004) Trichostatin A-like hydroxamate histone deacetylase inhibitors as therapeutic agents: toxicological point of view. Curr Med Chem 11:1629–1643
71. Marks PA, Xu WS (2009) Histone deacetylase inhibitors: Potential in cancer therapy. J Cell Biochem 107:600–608
72. Haggarty SJ, Koeller KM, Wong JC, Grozinger CM, Schreiber SL (2003) Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. Proc Natl Acad Sci USA 100:4389–4394
73. Zhang Y, Reinberg D (2001) Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev 15:2343–2360
74. Spannhoff A, Hauser AT, Heinke R, Sippl W, Jung M (2009) The emerging therapeutic potential of histone methyltransferase and demethylase inhibitors. ChemMedChem 4:1568–1582
75. Albert M, Helin K (2010) Histone methyltransferases in cancer. Semin Cell Dev Biol 21:209–220
76. Krivtsov AV, Armstrong SA (2007) MLL translocations, histone modifications and leukaemia stem-cell development. Nat Rev Cancer 7:823–833
77. Milne TA, Briggs SD, Brock HW et al (2002) MLL targets SET domain methyltransferase activity to Hox gene promoters. Mol Cell 10:1107–1117
78. Kleer CG, Cao Q, Varambally S et al (2003) EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc Natl Acad Sci USA 100:11606–11611
79. Varambally S, Dhanasekaran SM, Zhou M et al (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 419:624–629
80. Peters AH, O'Carroll D, Scherthan H et al (2001) Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. Cell 107:323–337
81. Varambally S, Cao Q, Mani RS et al (2008) Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. Science 322:1695–1699
82. Miranda TB, Cortez CC, Yoo CB et al (2009) DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation. Mol Cancer Ther 8:1579–1588
83. Spannhoff A, Sippl W, Jung M (2009) Cancer treatment of the future: inhibitors of histone methyltransferases. Int J Biochem Cell Biol 41:4–11
84. Tan J, Yang X, Zhuang L et al (2007) Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes Dev 21:1050–1063
85. Cloos PA, Christensen J, Agger K, Helin K (2008) Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. Genes Dev 22:1115–1140
86. Agger K, Christensen J, Cloos PA, Helin K (2008) The emerging functions of histone demethylases. Curr Opin Genet Dev 18:159–168
87. Tsukada Y, Fang J, Erdjument-Bromage H et al (2006) Histone demethylation by a family of JmjC domain-containing proteins. Nature 439:811–816
88. Whetstine JR, Nottke A, Lan F et al (2006) Reversal of histone lysine trimethylation by the JMJD2 family of histone demethylases. Cell 125:467–481
89. Meyer R, Wolf SS, Obendorf M (2007) PRMT2, a member of the protein arginine methyltransferase family, is a coactivator of the androgen receptor. J Steroid Biochem Mol Biol 107:1–14
90. Shi Y, Lan F, Matson C et al (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119:941–953
91. Lan F, Collins RE, De Cegli R et al (2007) Recognition of unmethylated histone H3 lysine 4 links BHC80 to LSD1-mediated gene repression. Nature 448:718–722
92. Shi Y (2007) Histone lysine demethylases: emerging roles in development, physiology and disease. Nat Rev Genet 8:829–833

93. Barrett A, Madsen B, Copier J et al (2002) PLU-1 nuclear protein, which is upregulated in breast cancer, shows restricted expression in normal human adult tissues: a new cancer/testis antigen? Int J Cancer 101:581–588
94. Kahl P, Gullotti L, Heukamp LC et al (2006) Androgen receptor coactivators lysine-specific histone demethylase 1 and four and a half LIM domain protein 2 predict risk of prostate cancer recurrence. Cancer Res 66:11341–11347
95. Sengupta N, Seto E (2004) Regulation of histone deacetylase activities. J Cell Biochem 93:57–67
96. de la Cruz CC, Kirmizis A, Simon MD, Isono K, Koseki H, Panning B (2007) The polycomb group protein SUZ12 regulates histone H3 lysine 9 methylation and HP1 alpha distribution. Chromosome Res 15:299–314
97. Santos-Rosa H, Caldas C (2005) Chromatin modifier enzymes, the histone code and cancer. Eur J Cancer 41:2381–2402
98. Wen B, Wu H, Shinkai Y, Irizarry RA, Feinberg AP (2009) Large histone H3 lysine 9 dimethylated chromatin blocks distinguish differentiated from embryonic stem cells. Nat Genet 41:246–250
99. Fahrner JA, Eguchi S, Herman JG, Baylin SB (2002) Dependence of histone modifications and gene expression on DNA hypermethylation in cancer. Cancer Res 62:7213–7218
100. Fraga MF, Ballestar E, Villar-Garea A et al (2005) Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet 37:391–400
101. Gerlo S, Haegeman G (2008) Vanden Berghe W. Transcriptional regulation of autocrine IL-6 expression in multiple myeloma cells. Cell Signal 20:1489–1496
102. Krejci J, Harnicarova A, Streitova D et al (2009) Epigenetics of multiple myeloma after treatment with cytostatics and gamma radiation. Leuk Res 33:1490–1498
103. Trussardi-Regnier A, Lavenus S, Gorisse MC, Dufer J (2009) Thalidomide alters nuclear architecture without ABCB1 gene modulation in drug-resistant myeloma cells. Int J Oncol 35:641–647
104. Deleu S, Lemaire M, Arts J et al (2009) The effects of JNJ-26481585, a novel hydroxamate-based histone deacetylase inhibitor, on the development of multiple myeloma in the 5T2MM and 5T33MM murine models. Leukemia 23:1894–1903
105. Catley L, Weisberg E, Kiziltepe T et al (2006) Aggresome induction by proteasome inhibitor bortezomib and alpha-tubulin hyperacetylation by tubulin deacetylase (TDAC) inhibitor LBH589 are synergistic in myeloma cells. Blood 108:3441–3449
106. Golay J, Cuppini L, Leoni F et al (2007) The histone deacetylase inhibitor ITF2357 has anti-leukemic activity in vitro and in vivo and inhibits IL-6 and VEGF production by stromal cells. Leukemia 21:1892–1900
107. Fandy TE, Shankar S, Ross DD, Sausville E, Srivastava RK (2005) Interactive effects of HDAC inhibitors and TRAIL on apoptosis are associated with changes in mitochondrial functions and expressions of cell cycle regulatory genes in multiple myeloma. Neoplasia 7:646–657
108. Mitsiades N, Mitsiades CS, Richardson PG et al (2003) Molecular sequelae of histone deacetylase inhibition in human malignant B cells. Blood 101:4055–4062
109. Kaiser M, Zavrski I, Sterz J et al (2006) The effects of the histone deacetylase inhibitor valproic acid on cell cycle, growth suppression and apoptosis in multiple myeloma. Haematologica 91:248–251
110. Catley L, Weisberg E, Tai YT et al (2003) NVP-LAQ824 is a potent novel histone deacetylase inhibitor with significant activity against multiple myeloma. Blood 102:2615–2622
111. Feng R, Oton A, Mapara MY, Anderson G, Belani C, Lentzsch S (2007) The histone deacetylase inhibitor, PXD101, potentiates bortezomib-induced anti-multiple myeloma effect by induction of oxidative stress and DNA damage. Br J Haematol 139:385–397
112. Feng R, Ma H, Hassig CA et al (2008) KD5170, a novel mercaptoketone-based histone deacetylase inhibitor, exerts antimyeloma effects by DNA damage and mitochondrial signaling. Mol Cancer Ther 7:1494–1505

113. De Bruyne E, Bos TJ, Schuit F et al (2010) IGF-1 suppresses Bim expression in multiple myeloma via epigenetic and posttranslational mechanisms. Blood 115:2430–2440
114. Khan SB, Maududi T, Barton K, Ayers J, Alkan S (2004) Analysis of histone deacetylase inhibitor, depsipeptide (FR901228), effect on multiple myeloma. Br J Haematol 125:156–161
115. Lavelle D, Chen YH, Hankewych M, DeSimone J (2001) Histone deacetylase inhibitors increase p21(WAF1) and induce apoptosis of human myeloma cell lines independent of decreased IL-6 receptor expression. Am J Hematol 68:170–178
116. Gomez-Benito M, Martinez-Lorenzo MJ, Anel A, Marzo I, Naval J (2007) Membrane expression of DR4, DR5 and caspase-8 levels, but not Mcl-1, determine sensitivity of human myeloma cells to Apo2L/TRAIL. Exp Cell Res 313:2378–2388
117. Schwartz C, Palissot V, Aouali N et al (2007) Valproic acid induces non-apoptotic cell death mechanisms in multiple myeloma cell lines. Int J Oncol 30:573–582
118. Mitsiades CS, Mitsiades NS, McMullan CJ et al (2004) Transcriptional signature of histone deacetylase inhibition in multiple myeloma: biological and clinical implications. Proc Natl Acad Sci USA 101:540–545
119. De Bruyne E, Bos TJ, Asosingh K et al (2008) Epigenetic silencing of the tetraspanin CD9 during disease progression in multiple myeloma cells and correlation with survival. Clin Cancer Res 14:2918–2926
120. Atadja P, Hsu M, Kwon P, Trogani N, Bhalla K, Remiszewski S (2004) Molecular and cellular basis for the anti-proliferative effects of the HDAC inhibitor LAQ824. Novartis Found Symp 259:249–266 (discussion 66–8, 85–8)
121. Deleu S, Lemaire M, Arts J et al (2009) Bortezomib alone or in combination with the histone deacetylase inhibitor JNJ-26481585: effect on myeloma bone disease in the 5T2MM murine model of myeloma. Cancer Res 69:5307–5311
122. Hubbert C, Guardiola A, Shao R et al (2002) HDAC6 is a microtubule-associated deacetylase. Nature 417:455–458
123. Wickner S, Maurizi MR, Gottesman S (1999) Posttranslational quality control: folding, refolding, and degrading proteins. Science 286:1888–1893
124. Bennett EJ, Bence NF, Jayakumar R, Kopito RR (2005) Global impairment of the ubiquitin-proteasome system by nuclear or cytoplasmic protein aggregates precedes inclusion body formation. Mol Cell 17:351–365
125. Kopito RR (2000) Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol 10:524–530
126. Lee JY, Koga H, Kawaguchi Y et al (2010) HDAC6 controls autophagosome maturation essential for ubiquitin-selective quality-control autophagy. EMBO J 29:969–980
127. Hideshima T, Bradner JE, Wong J et al (2005) Small-molecule inhibition of proteasome and aggresome function induces synergistic antitumor activity in multiple myeloma. Proc Natl Acad Sci USA 102:8567–8572
128. Nawrocki ST, Carew JS, Maclean KH et al (2008) Myc regulates aggresome formation, the induction of Noxa, and apoptosis in response to the combination of bortezomib and SAHA. Blood 112:2917–2926
129. Bali P, Pranpat M, Bradner J et al (2005) Inhibition of histone deacetylase 6 acetylates and disrupts the chaperone function of heat shock protein 90: a novel basis for antileukemia activity of histone deacetylase inhibitors. J Biol Chem 280:26729–26734
130. Jagannath S, Dimopoulos MA, Lonial S (2010) Combined proteasome and histone deacetylase inhibition: a promising synergy for patients with relapsed/refractory multiple myeloma. Leuk Res 34(9):1111–1118
131. Pei XY, Dai Y, Grant S (2004) Synergistic induction of oxidative injury and apoptosis in human multiple myeloma cells by the proteasome inhibitor bortezomib and histone deacetylase inhibitors. Clin Cancer Res 10:3839–3852
132. Rahmani M, Yu C, Dai Y et al (2003) Coadministration of the heat shock protein 90 antagonist 17-allylamino- 17-demethoxygeldanamycin with suberoylanilide hydroxamic acid or sodium butyrate synergistically induces apoptosis in human leukemia cells. Cancer Res 63:8420–8427

133. Rao R, Fiskus W, Yang Y et al (2008) HDAC6 inhibition enhances 17-AAG–mediated abrogation of hsp90 chaperone function in human leukemia cells. Blood 112:1886–1893
134. Stuhmer T, Arts J, Chatterjee M et al (2010) Preclinical anti-myeloma activity of the novel HDAC-inhibitor JNJ-26481585. Br J Haematol 149(4):529–536
135. Maiso P, Carvajal-Vergara X, Ocio EM et al (2006) The histone deacetylase inhibitor LBH589 is a potent antimyeloma agent that overcomes drug resistance. Cancer Res 66:5781–5789
136. Ambros V (2004) The functions of animal microRNAs. Nature 431:350–355
137. Pillai RS, Bhattacharyya SN, Filipowicz W (2007) Repression of protein synthesis by miRNAs: how many mechanisms? Trends Cell Biol 17:118–126
138. Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 9:102–114
139. Saetrom P, Snove O Jr, Rossi JJ (2007) Epigenetics and microRNAs. Pediatr Res 61:17R–23R
140. Girard M, Jacquemin E, Munnich A, Lyonnet S, Henrion-Caude A (2008) miR-122, a paradigm for the role of microRNAs in the liver. J Hepatol 48:648–656
141. Tang X, Gal J, Zhuang X, Wang W, Zhu H, Tang G (2007) A simple array platform for microRNA analysis and its application in mouse tissues. RNA 13(10):1803–1822
142. Varnholt H (2008) The role of microRNAs in primary liver cancer. Ann Hepatol 7:104–113
143. Esquela-Kerscher A, Slack FJ (2006) Oncomirs – microRNAs with a role in cancer. Nat Rev Cancer 6:259–269
144. Kent OA, Mendell JT (2006) A small piece in the cancer puzzle: microRNAs as tumor suppressors and oncogenes. Oncogene 25:6188–6196
145. Williams AE (2008) Functional aspects of animal microRNAs. Cell Mol Life Sci 65:545–562
146. Lu J, Getz G, Miska EA et al (2005) MicroRNA expression profiles classify human cancers. Nature 435:834–838
147. Rosenfeld N, Aharonov R, Meiri E et al (2008) MicroRNAs accurately identify cancer tissue origin. Nat Biotechnol 26:462–469
148. Calin GA, Dumitru CD, Shimizu M et al (2002) Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci USA 99:15524–15529
149. Ruan K, Fang X, Ouyang G (2009) MicroRNAs: novel regulators in the hallmarks of human cancer. Cancer Lett 285:116–126
150. Loffler D, Brocke-Heidrich K, Pfeifer G et al (2007) Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. Blood 110:1330–1333
151. Pichiorri F, Suh SS, Ladetto M et al (2008) MicroRNAs regulate critical genes associated with multiple myeloma pathogenesis. Proc Natl Acad Sci USA 105:12885–12890
152. Roccaro AM, Sacco A, Thompson B et al (2009) MicroRNAs 15a and 16 regulate tumor proliferation in multiple myeloma. Blood 113:6669–6680
153. Corthals SL, Jongen-Lavrencic M, de Knegt Y et al (2010) Micro-RNA-15a and micro-RNA-16 expression and chromosome 13 deletions in multiple myeloma. Leuk Res 34(5):677–681
154. Lerner M, Harada M, Loven J et al (2009) DLEU2, frequently deleted in malignancy, functions as a critical host gene of the cell cycle inhibitory microRNAs miR-15a and miR-16-1. Exp Cell Res 315:2941–2952
155. Gutierrez NC, Sarasquete ME, Misiewicz-Krzeminska I et al (2010) Deregulation of microRNA expression in the different genetic subtypes of multiple myeloma and correlation with gene expression profiling. Leukemia 24:629–637
156. Lionetti M, Agnelli L, Mosca L et al (2009) Integrative high-resolution microarray analysis of human myeloma cell lines reveals deregulated miRNA expression associated with allelic imbalances and gene expression profiles. Genes Chromosomes Cancer 48:521–531
157. Lionetti M, Biasiolo M, Agnelli L et al (2009) Identification of microRNA expression patterns and definition of a microRNA/mRNA regulatory network in distinct molecular groups of multiple myeloma. Blood 114:e20–e26

158. Zhou Y, Chen L, Barlogie B et al (2010) High-risk myeloma is associated with global elevation of miRNAs and overexpression of EIF2C2/AGO2. Proc Natl Acad Sci USA 107:7904–7909
159. Diederichs S, Haber DA (2007) Dual role for argonautes in microRNA processing and post-transcriptional regulation of microRNA expression. Cell 131:1097–1108
160. Liu J, Carmell MA, Rivas FV et al (2004) Argonaute2 is the catalytic engine of mammalian RNAi. Science 305:1437–1441
161. Shaughnessy JD Jr, Zhan F, Burington BE et al (2007) A validated gene expression model of high-risk multiple myeloma is defined by deregulated expression of genes mapping to chromosome 1. Blood 109:2276–2284
162. Tamaru H, Selker EU (2001) A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. Nature 414:277–283
163. Tamaru H, Zhang X, McMillen D et al (2003) Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in Neurospora crassa. Nat Genet 34:75–79
164. Dong KB, Maksakova IA, Mohn F et al (2008) DNA methylation in ES cells requires the lysine methyltransferase G9a but not its catalytic activity. EMBO J 27:2691–2701
165. Ikegami K, Iwatani M, Suzuki M et al (2007) Genome-wide and locus-specific DNA hypomethylation in G9a deficient mouse embryonic stem cells. Genes Cells 12:1–11
166. Tachibana M, Matsumura Y, Fukuda M, Kimura H, Shinkai Y (2008) G9a/GLP complexes independently mediate H3K9 and DNA methylation to silence transcription. EMBO J 27:2681–2690
167. Esteve PO, Chin HG, Smallwood A et al (2006) Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication. Genes Dev 20:3089–3103
168. Kondo Y (2009) Epigenetic cross-talk between DNA methylation and histone modifications in human cancers. Yonsei Med J 50:455–463
169. Smallwood A, Esteve PO, Pradhan S, Carey M (2007) Functional cooperation between HP1 and DNMT1 mediates gene silencing. Genes Dev 21:1169–1178
170. Wozniak RJ, Klimecki WT, Lau SS, Feinstein Y, Futscher BW (2007) 5-Aza-2′-deoxycytidine-mediated reductions in G9A histone methyltransferase and histone H3K9 di-methylation levels are linked to tumor suppressor gene reactivation. Oncogene 26:77–90
171. Okitsu CY, Hsieh CL (2007) DNA methylation dictates histone H3K4 methylation. Mol Cell Biol 27:2746–2757
172. Weber M, Hellmann I, Stadler MB et al (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet 39:457–466
173. Ooi SK, Qiu C, Bernstein E et al (2007) DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. Nature 448:714–717
174. Hotz HR, Peters AH (2009) Protein demethylation required for DNA methylation. Nat Genet 41:10–11
175. Bernstein BE, Mikkelsen TS, Xie X et al (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125:315–326
176. Mikkelsen TS, Ku M, Jaffe DB et al (2007) Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature 448:553–560
177. Kondo Y, Shen L, Cheng AS et al (2008) Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation. Nat Genet 40:741–750
178. Bernstein BE, Meissner A, Lander ES (2007) The mammalian epigenome. Cell 128:669–681
179. Sparmann A, van Lohuizen M (2006) Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer 6:846–856
180. Dellino GI, Schwartz YB, Farkas G, McCabe D, Elgin SC, Pirrotta V (2004) Polycomb silencing blocks transcription initiation. Mol Cell 13:887–893
181. Levine SS, Weiss A, Erdjument-Bromage H, Shao Z, Tempst P, Kingston RE (2002) The core of the polycomb repressive complex is compositionally and functionally conserved in flies and humans. Mol Cell Biol 22:6070–6078
182. Shao Z, Raible F, Mollaaghababa R et al (1999) Stabilization of chromatin structure by PRC1, a Polycomb complex. Cell 98:37–46

183. Simon JA, Kingston RE (2009) Mechanisms of polycomb gene silencing: knowns and unknowns. Nat Rev Mol Cell Biol 10:697–708
184. Ben-Porath I, Thomson MW, Carey VJ et al (2008) An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet 40:499–507
185. Yu J, Rhodes DR, Tomlins SA et al (2007) A polycomb repression signature in metastatic prostate cancer predicts cancer outcome. Cancer Res 67:10657–10663
186. Bachmann IM, Halvorsen OJ, Collett K et al (2006) EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J Clin Oncol 24:268–273
187. Collett K, Eide GE, Arnes J et al (2006) Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer. Clin Cancer Res 12:1168–1174
188. Croonquist PA, Van Ness B (2005) The polycomb group protein enhancer of zeste homolog 2 (EZH 2) is an oncogene that influences myeloma cell growth and the mutant ras phenotype. Oncogene 24:6269–6280
189. Kalushkova A, Fryknas M, Lemaire M et al (2010) Polycomb target genes are silenced in multiple myeloma. PLoS One 5(7):e11483
190. Bracken AP, Pasini D, Capra M, Prosperini E, Colli E, Helin K (2003) EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. EMBO J 22:5323–5335
191. Otte AP, Kwaks TH (2003) Gene repression by Polycomb group protein complexes: a distinct complex for every occasion? Curr Opin Genet Dev 13:448–454
192. Sander S, Bullinger L, Klapproth K et al (2008) MYC stimulates EZH2 expression by repression of its negative regulator miR-26a. Blood 112:4202–4212
193. van der Vlag J, Otte AP (1999) Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. Nat Genet 23:474–478
194. Fiskus W, Pranpat M, Balasis M et al (2006) Histone deacetylase inhibitors deplete enhancer of zeste 2 and associated polycomb repressive complex 2 proteins in human acute leukemia cells. Mol Cancer Ther 5:3096–3104
195. Ohm JE, McGarvey KM, Yu X et al (2007) A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat Genet 39:237–242
196. Schlesinger Y, Straussman R, Keshet I et al (2007) Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet 39:232–236
197. Vire E, Brenner C, Deplus R et al (2006) The Polycomb group protein EZH2 directly controls DNA methylation. Nature 439:871–874
198. Widschwendter M, Fiegl H, Egle D et al (2007) Epigenetic stem cell signature in cancer. Nat Genet 39:157–158
199. Negishi M, Saraya A, Miyagi S et al (2007) Bmi1 cooperates with Dnmt1-associated protein 1 in gene silencing. Biochem Biophys Res Commun 353:992–998
200. McCabe MT, Lee EK, Vertino PM (2009) A multifactorial signature of DNA sequence and polycomb binding predicts aberrant CpG island methylation. Cancer Res 69:282–291
201. Gal-Yam EN, Egger G, Iniguez L et al (2008) Frequent switching of Polycomb repressive marks and DNA hypermethylation in the PC3 prostate cancer cell line. Proc Natl Acad Sci USA 105:12979–12984
202. McGarvey KM, Greene E, Fahrner JA, Jenuwein T, Baylin SB (2007) DNA methylation and complete transcriptional silencing of cancer genes persist after depletion of EZH2. Cancer Res 67:5097–5102
203. Brito JL, Walker B, Jenner M et al (2009) MMSET deregulation affects cell cycle progression and adhesion regulons in t(4;14) myeloma plasma cells. Haematologica 94:78–86
204. van Haaften G, Dalgliesh GL, Davies H et al (2009) Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. Nat Genet 41:521–523
205. Kuehl WM, Bergsagel PL (2002) Multiple myeloma: evolving genetic events and host interactions. Nat Rev Cancer 2:175–187

206. Fiskus W, Buckley K, Rao R et al (2009) Panobinostat treatment depletes EZH2 and DNMT1 levels and enhances decitabine mediated de-repression of JunB and loss of survival of human acute leukemia cells. Cancer Biol Ther 8:939–950
207. Milutinovic S, D'Alessio AC, Detich N, Szyf M (2007) Valproate induces widespread epigenetic reprogramming which involves demethylation of specific genes. Carcinogenesis 28:560–571
208. Ou JN, Torrisani J, Unterberger A et al (2007) Histone deacetylase inhibitor Trichostatin A induces global and gene-specific DNA demethylation in human cancer cell lines. Biochem Pharmacol 73:1297–1307
209. Wu LP, Wang X, Li L et al (2008) Histone deacetylase inhibitor depsipeptide activates silenced genes through decreasing both CpG and H3K9 methylation on the promoter. Mol Cell Biol 28:3219–3235
210. Xiong Y, Dowdy SC, Podratz KC et al (2005) Histone deacetylase inhibitors decrease DNA methyltransferase-3B messenger RNA stability and down-regulate de novo DNA methyltransferase activity in human endometrial cells. Cancer Res 65:2684–2689
211. McGarvey KM, Fahrner JA, Greene E, Martens J, Jenuwein T, Baylin SB (2006) Silenced tumor suppressor genes reactivated by DNA demethylation do not return to a fully euchromatic chromatin state. Cancer Res 66:3541–3549
212. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 21:103–107
213. Klisovic MI, Maghraby EA, Parthun MR et al (2003) Depsipeptide (FR 901228) promotes histone acetylation, gene transcription, apoptosis and its activity is enhanced by DNA methyltransferase inhibitors in AML1/ETO-positive leukemic cells. Leukemia 17:350–358
214. Tong WG, Wei Y, Stevenson W et al (2010) Preclinical antileukemia activity of JNJ-26481585, a potent second-generation histone deacetylase inhibitor. Leuk Res 34:221–228
215. Yang H, Hoshino K, Sanchez-Gonzalez B, Kantarjian H, Garcia-Manero G (2005) Antileukemia activity of the combination of 5-aza-2′-deoxycytidine with valproic acid. Leuk Res 29:739–748
216. Benetti R, Gonzalo S, Jaco I et al (2008) A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. Nat Struct Mol Biol 15:998
217. Ting AH, Suzuki H, Cope L et al (2008) A requirement for DICER to maintain full promoter CpG island hypermethylation in human cancer cells. Cancer Res 68:2570–2575
218. Chuang JC, Jones PA (2007) Epigenetics and microRNAs. Pediatr Res 61:24R–29R
219. Fabbri M, Garzon R, Cimmino A et al (2007) MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci USA 104:15805–15810
220. Garzon R, Liu S, Fabbri M et al (2009) MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. Blood 113:6411–6418
221. Lehmann U, Hasemeier B, Christgen M et al (2008) Epigenetic inactivation of microRNA gene hsa-mir-9-1 in human breast cancer. J Pathol 214:17–24
222. Datta J, Kutay H, Nasser MW et al (2008) Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis. Cancer Res 68:5049–5058
223. Noonan EJ, Place RF, Pookot D et al (2009) miR-449a targets HDAC-1 and induces growth arrest in prostate cancer. Oncogene 28:1714–1724
224. Tuddenham L, Wheeler G, Ntounia-Fousara S et al (2006) The cartilage specific microRNA-140 targets histone deacetylase 4 in mouse cells. FEBS Lett 580:4214–4217
225. Friedman JM, Liang G, Liu CC et al (2009) The putative tumor suppressor microRNA-101 modulates the cancer epigenome by repressing the polycomb group protein EZH2. Cancer Res 69:2623–2629
226. Sander S, Bullinger L, Karlsson A et al (2005) Comparative genomic hybridization on mouse cDNA microarrays and its application to a murine lymphoma model. Oncogene 24:6101–6107

227. Saito Y, Liang G, Egger G et al (2006) Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 9:435–443
228. Scott GK, Mattie MD, Berger CE, Benz SC, Benz CC (2006) Rapid alteration of microRNA levels by histone deacetylase inhibition. Cancer Res 66:1277–1281
229. Lujambio A, Ropero S, Ballestar E et al (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. Cancer Res 67:1424–1429
230. Agirre X, Vilas-Zornoza A, Jimenez-Velasco A et al (2009) Epigenetic silencing of the tumor suppressor microRNA Hsa-miR-124a regulates CDK6 expression and confers a poor prognosis in acute lymphoblastic leukemia. Cancer Res 69:4443–4453
231. Lujambio A, Calin GA, Villanueva A et al (2008) A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci USA 105:13556–13561
232. Fazi F, Racanicchi S, Zardo G et al (2007) Epigenetic silencing of the myelopoiesis regulator microRNA-223 by the AML1/ETO oncoprotein. Cancer Cell 12:457–466
233. Meng F, Wehbe-Janek H, Henson R, Smith H, Patel T (2008) Epigenetic regulation of microRNA-370 by interleukin-6 in malignant human cholangiocytes. Oncogene 27:378–386
234. Grady WM, Parkin RK, Mitchell PS et al (2008) Epigenetic silencing of the intronic microRNA hsa-miR-342 and its host gene EVL in colorectal cancer. Oncogene 27:3880–3888
235. Saito Y, Friedman JM, Chihara Y, Egger G, Chuang JC, Liang G (2009) Epigenetic therapy upregulates the tumor suppressor microRNA-126 and its host gene EGFL7 in human cancer cells. Biochem Biophys Res Commun 379:726–731
236. Todoerti K, Barbui V, Pedrini O et al (2010) Pleiotropic anti-myeloma activity of ITF2357: inhibition of interleukin-6 receptor signaling and repression of miR-19a and miR-19b. Haematologica 95:260–269
237. Chim CS, Wong KY, Qi Y et al (2010) Epigenetic inactivation of the miR-34a in hematological malignancies. Carcinogenesis 31:745–750

Chapter 15
Targeting Multiple Myeloma Tumor Angiogenesis: Focus on VEGF

Klaus Podar and Kenneth C. Anderson

Abstract The role of angiogenesis in solid tumors but also in hematologic malignancies including multiple myeloma (MM) is now well established. Research on angiogenesis in general and vascular endothelial growth factor (VEGF), in particular, is a major focus in biomedicine. Derived antiangiogenic therapeutics including the monoclonal anti- VEGF antibody bevazicumab; and the second- generation multitargeted receptor kinase inhibitors (RTKIs) sorafenib, sunitinib, and pazopanib have fundamentally changed treatment strategies in progressed solid tumors over the past decade. In MM, increased microvessel density (MVD) within the bone marrow of MM patients correlates with disease progression and poor prognosis. The therapeutic success of thalidomide and the novel agents bortezomib and lenalidomide in MM treatment is based, at least in part, on their activity against VEGF production and secretion and related effects within the BM microenvironment. Based on these observations, several preclinical and clinical studies are ongoing to evaluate strategies, which either directly or indirectly target VEGF and VEGF receptors and the "vascular niche" in order to improve MM patient outcome.

K. Podar (✉)
National Center for Tumor Diseases (NCT)/German Cancer Research Center and University of Heidelberg, Heidelberg, Germany

Department of Medical Oncology,
Dana-Farber Cancer Institute, Harvard Medical School,
Boston, MA, USA
e-mail: klaus.podar@med.uni-heidelberg.de; klaus.podar@nct-heidelberg.de

K.C. Anderson
Department of Medical Oncology,
Dana-Farber Cancer Institute, Harvard Medical School,
Boston, MA, USA

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_15,

15.1 Introduction

A new era of research to identify pro- and antiangiogenic molecules and develop derived targeted therapies was triggered by Dr. Judah Folkman's seminal postulation in 1971 that angiogenesis is required for tumor growth and progression and may represent a new target for cancer therapy [1]. After more than 30 years, it is well established that angiogenesis plays not only a pivotal role in solid tumors but also in hematologic malignancies [2, 3]. Today, research on the formation of new blood vessels (angiogenesis), in general, and VEGF, in particular, is a major focus in biomedicine, and has led to the clinical approval of the monoclonal anti-VEGF antibody bevazicumab; and the second-generation multitargeted receptor kinase inhibitors (RTKIs) sorafenib, sunitinib, and pazopanib in metastatic colorectal cancer, hepatocellular carcinoma (HCC), non-small cell lung cancer (NSCLC), glioblastoma multiforme (GBM) renal cell carcinoma (RCC), and gastrointestinal stroma tumor (GIST).

In the first well-documented case of multiple myeloma (MM), Samuel Solly described the disease as a "morbid action of the blood vessels in which the earthy matter of the bone is absorbed and thrown out by the kidneys in the urine." Solly is thereby the first to anticipate the role of the bone marrow microenvironment and angiogenesis in MM pathogenesis. However, it was not until 150 years later that Vacca et al. reported for the first time in 1994 increased microvessel density (MVD) within the BM of MM versus monoclonal gammopathy of undetermined significance (MGUS) patients and in active (diagnosis, relapse, leukemic phase) versus non-active (complete/objective response, plateau) MM [4]. Importantly, these findings correlate with disease progression and poor prognosis [5, 6]. Moreover, BM MVD at the time of initial diagnosis is an important prognostic factor for median overall survival (OS) and median progression-free survival (PFS) in patients undergoing autologous transplantation as frontline therapy for MM [7–9]. These data, coupled with antiangiogenic properties of thalidomide recognized by D'Amato and Folkman [10], provided the rationale for the therapeutic use of thalidomide in MM. Indeed, the landmark trial by Singhal and Barlogie demonstrated responses in 30% of MM patients whose disease was refractory to conventional and high-dose therapy [11].

A significant decrease of MVD further supported an antiangiogenic effect of thalidomide, and suggested the association of angiogenesis and primary resistance to therapy of more aggressive disease [12, 13]. Subsequent studies demonstrated that increased BM MVD is mediated by an unbalance of normally finely tuned expression of pro- and antiangiogenic molecules [14–18].

Here we review up-to-date insights on the MM BM microenvironment and the "vascular niche" in particular. Moreover, we summarize recent derived approaches to target BM angiogenesis and vascular endothelial growth factor (VEGF), the key angiogenic activator in tumor angiogenesis, using novel therapeutic strategies.

15.2 The Vascular Niche Within the Multiple Myeloma Bone Marrow Microenvironment

Following the discovery of the therapeutic value of thalidomide in MM, a multitude of preclinical studies by us and others have highlighted the pivotal role of the BM microenvironment in MM pathogenesis and identified additional new therapeutic targets. The therapeutic success of derived novel agents, e.g., bortezomib and lenalidomide, even in relapsed/refractory disease is, at least in part, due to their activity against BM microenvironment-derived (e.g., antiangiogenic) effects on MM cells, confirming the therapeutic promise of targeting MM–BM milieu interactions.

The BM environment located within the protective coat of mineralized bone consists of hematopoietic and non-hematopoietic cells, as well as an extracellular and liquid compartment. It is organized in a complex three-dimensional architecture of sub-microenvironments ("niches"). Under physiologic conditions, these compartments are highly organized by cell–matrix and cell–cell interactions within a regulative liquid milieu and support normal hematopoiesis. In MM, the balanced homeostasis between these BM compartments is disrupted and supports MM cell proliferation, survival, migration and drug resistance via activation of various signaling pathways [19].

The "vascular niche," in particular, is comprised of vasculature forming a conduit between the "encapsulated chamber" of the BM and the peripheral circulation to enable MM cells both to leave the osteoblastic niche and enter the vascular system via transendothelial migration (mobilization), and to return to the BM via homing [19]. Functionally, it is now clear that the vascular niche is not only a transfer system for mature blood cells to the peripheral circulation, but also a site required for the differentiation and maturation of hematopoietic progenitors via both the production and secretion of various cytokines and growth factors, as well as direct contact. Importantly, hematopoietic cells in turn prolong survival of BM endothelial cells by secreting endothelial cell survival factors, such as VEGF.

The complexity of angiogenesis characterized by sprouting and co-option [20] of neighboring pre-existing vessels is further enhanced by VEGF-induced recruitment of highly proliferative circulatory endothelial progenitors (CEPs) from the BM, hematopoietic stem cells (HSCs), progenitor cells, monocytes, and macrophages [21]. Remarkably, also tumor cells can act as endothelial cells and form functional avascular blood conduits or mosaic blood vessels [21–27].

The imbalance of pro-angiogenic regulators, i.e. VEGF, accounts for an abnormal structure of tumor vessels resulting in chaotic, variable blood flow and vessel leakiness, and thereby lowering drug delivery and selecting for more malignant tumor cells [28–31]. Indeed, Vacca et al. also showed the existence of a heterogeneous population of endothelial cells derived from patient BM (MMECs) which differs from its normal counterpart in morphology, antigen expression, and function, resulting in tortuous, uneven vessels with profuse branching and shunts. These findings indicate that MMECs facilitate tumor cell growth, invasion, and dissemination more than HUVECs [32].

15.3 The Role of VEGF in MM

VEGF and its receptors are expressed both by stromal cells and a variety of tumor cells, including MM cells. VEGF within the BM microenvironment of MM is overexpressed and secreted predominantly by the clonal plasma cells. One main regulatory circuit of MM cell-derived VEGF secretion is represented by the functional interaction of interleukin-6 (IL-6) and VEGF. Specifically, MM cells trigger IL-6 secretion by BM stromal cells (BMSCs). In turn, BMSC-derived IL-6 promotes proliferation, survival, and VEGF production in plasma cells [15, 33]. Both IL-6 and VEGF secretion are additionally induced by CD40 activation of tumor cells, as well as by a variety of other growth factors and cytokines present in the BM microenvironment, including TNFα and IL-1 [34] [35]. VEGF-mediated paracrine effects on endothelial cells are mediated via VEGF receptor-2 (VEGFR-2), and VEGF-mediated paracrine effects on stromal cells as well as autocrine effects on tumor cells are mediated via VEGFR-1. Besides stimulating endothelial cell proliferation and migration, VEGF also enhances endothelial expression and activation of adhesion proteins (e.g., integrins) and stimulates secretion of proteases and ECM components, thereby enabling the remodeling of extracellular matrix (ECM) structures and modulating tumor cell–tumor cell as well as tumor cell–ECM interactions [36]. Although VEGF represents the predominant angiogenic factor, the concerted function of additional pro-angiogenic molecules is required for angiogenesis. These factors include basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), placenta-like growth factor (PlGF), transforming growth factor-β, platelet-derived endothelial growth factor (PD-EGF), angiopoetin-1, and pleiotrophin. While VEGF directly stimulates endothelial cell growth, the other pro-angiogenic factors predominantly recruit supporting cells such as pericytes and smooth muscle cells, as well as proteins of the ECM. Inducers of angiogenesis in the MM BM microenvironment include IL-6, TNF-α, FGF-2, HGF, IGF-1, MIP-1, MCP-1, and SDF-1 [37]. Antiangiogenic factors including angiostatin, endostatin, and thrombostatin finely tune angiogenesis by regulating expression of the above activators [38, 39].

In addition to its role in angiogenesis, VEGF triggers a multitude of additional sequelae, which are likely to contribute to the clinical features of MM including direct tumor cell survival, migration and moderate proliferation, immune suppression and lytic bone lesions [40, 41]. It therefore represents a potential new therapeutic target in MM.

15.4 Strategies to Directly and Indirectly Targeting Angiogenesis and VEGF-signaling Sequelae

Approaches to disrupt the VEGF/VEGF receptor signaling pathways range from small molecule VEGF receptor inhibitors to biological agents including soluble receptors, anti-VEGF and anti-VEGF receptor antibodies, and VEGF transcription inhibitors. Although avastin is an effective medication and studies testing the

VEGF-trap or VEGFR-targeting antibodies are promising, drug resistance always develops likely due to targeting a single tumorigenic pathway. Indeed, extended blockade of VEGF alone results in tumor revascularization, dependent on other pro-angiogenic factors such as FGF [42]. Small-molecule inhibitors therefore have the advantage of being both orally available and more promiscuous in target inhibition.

15.4.1 Bevacizumab (Avastin™)

A recent milestone in cancer therapy was the approval of bevacizumab, a humanized monoclonal antibody against VEGF, by the US Food and Drug Administration as first-line therapy for metastatic colorectal cancer in February 2004. Bevacizumab represents the first cancer drug specifically designed to target VEGF. Mechanisms of action include not only inhibition of tumor angiogenesis but also tumor vessel normalization, and inhibition of vascular recruitment of circulatory endothelial cells (CECs) and endothelial progenitor cells (EPCs). Indeed, bevacizumab-induced increases of the systemic fraction of tumor endothelial cells with pericyte coverage may reflect the dropout of immature endothelial cells and potentially provide a novel biomarker [43]. Following metastatic colorectal cancer, the effectiveness of bevacizumab has also been demonstrated in several other tumors, however, only when combined with conventional chemotherapy [44]. To date, more than 300 clinical trials are evaluating bevacizumab in a variety of solid and hematologic malignancies, including MM (http://www.clinicaltrials.gov/). For example, ongoing trials in MM evaluate the efficacy of bevacizumab either in combination with lenalidomide and dexamethasone or in combination with bortezomib in relapsed or refractory MM.

15.4.2 VEGF-trap, HuMV833 and VEGFR antibodies

VEGF-trap (Aflibercept, Regeneron Pharmaceuticals, Tarrytown, NY) is a soluble decoy receptor protein consisting of a hybrid Fc construct in which domain 2 of VEGFR-1 is fused to domain 3 of the VEGFR-2 [45, 46]. It has high affinity for all isoforms of VEGF-A and causes regression of co-opted vessels in a model of neuroblastoma [47]. Several clinical phase II/III trials testing the VEGF-trap in solid malignancies including prostate cancer, NSCLC, colorectal, and pancreatic cancer as well as a clinical phase II trial in patients with stage II or stage III MM which have relapsed or did not respond to previous treatment are ongoing (http://clinicaltrials.gov/). Similarly, HuMV833 is a humanized monoclonal IgG antibody that binds VEGF-A isoforms VEGF121 and VEGF165 with antitumor activity in human tumor xenograft models [48, 49]. Further antibodies against VEGFR-1 or VEGFR-2 (IMC-18 F1, IMC-1121B ImClone, New York, NY) are under preclinical and clinical investigation.

15.4.3 Sorafenib and Sunitinib

Two RTK inhibitors have now been approved by the FDA: [1] sorafenib (Nexavar, BAY43-9006, Bayer HealthCare Pharmaceuticals and Onyx Pharmaceuticals) [50, 51], which targets VEGFR2, VEGFR-3, Raf, PDGFRβ, Flt3 and c-Kit for the treatment of advanced RCC in 2005 and for the treatment of unresectable hepatocellular carcinoma (HCC) in 2007, with advanced clinical studies in NSCLC and melanoma; and [2] sunitinib (Sutent, SU11248, Pfizer) [52], which targets VEGFR2, PDGFRα/β, c-Kit, Flt3, RET for first-line and second-line therapy of metastatic RCC [53, 54] and for treatment of nongastrointestinal stromal tumor sarcomas [55] in 2006, with advanced clinical studies in breast, colorectal, and lung cancer.

In MM, a clinical phase II trial evaluated the efficacy of sunitinib in relapsed or refractory MM. Results are pending. Most recent data demonstrated significant sorafenib-induced preclinical anti-MM activity and synergized with common anti-MM drugs [56]. A variety of ongoing clinical trials evaluate the therapeutic value of sorafenib alone or in combination with bortezomib, lenalidomide, and dexamethasone; or everolimus.

Side effects include hand–foot skin reaction, diarrhea, alopecia, fatigue, nausea, and hypertension for sorafenib; and fatigue, nausea, diarrhea, and vomiting for sunitinib.

15.4.4 Vatalanib (PTK787/ZK222584)

Vatalanib (Novartis Pharmaceuticals, East Hanover, NJ) is an orally available tyrosine kinase inhibitor which binds to the ATP-binding sites of VEGF receptors [57, 58]. In MM, we have reported that vatalanib [1] acts directly on MM cells to inhibit VEGF-induced MM cell growth and migration and [2] inhibits paracrine IL-6-mediated MM cell growth in the BM milieu [59]. A trial testing vatalanib as post-transplant maintenance therapy in patients with MM has now been completed. Results show that vatalanib failed to show any significant decrease of microvessel count in BM biopsies assessed by immunohistochemistry prior to vatalanib initiation, after three cycles of therapy, and at the end of therapy. Also there was no change in BM cellularity and plasma cell count noted. In summary, the study failed to demonstrate a clinical benefit for maintenance therapy with vatalanib in the post-ASCT setting for MM [60].

15.4.5 Pazopanib (GW786034B)

Pazopanib (GlaxoSmithKline, Research Triangle Park, NC) is an orally available, small-molecule tyrosine kinase inhibitor of VEGF-receptor-1, -2, -3 with IC50s of 10, 30, and 47 nM, respectively [61, 62]. An initial non-randomized, dose-escalation phase I study with pazopanib (GSK-VEG10003) showed stable disease or partial

responses in relapsed/refractory patients with renal cell (RCC), Hurthle cell, neuroendocrine, GIST, and adeno-lung carcinoma, as well as chondrosarcoma, leiomyosarcoma, and melanoma with manageable side effects [63]. Based on a phase III clinical trial, pazopanib was FDA approved in October 2009 for the treatment of locally advanced or metastatic RCC [64]. Ongoing studies are evaluating pazopanib for the treatment of a variety of other malignancies including breast cancer, ovarian cancer, cervical cancer, NSCLC, colorectal cancer, and head and neck cancer.

In MM, pazopanib inhibits VEGF-triggered signaling pathways in tumor and endothelial cells, both in vitro and in vivo. Moreover, low-dose pazopanib demonstrated synergistic cytotoxicity with conventional (melphalan) and novel (bortezomib, immunomodulatory drugs) therapies [65]. These preclinical studies provided the rationale for clinical evaluation of pazopanib. In a clinical phase II study, pazopanib did not demonstrate activity for relapsed or refractory MM [66].

15.4.6 Other VEGF Inhibitors

Significant efficacy of RTK inhibitors including small molecules SU5416 (Sugen) [67–69], SU11248 (Sugen) [52], AG013676 and CP-547,632 (Pfizer) [70], ZD6474 (Vandetanib, ZACTIMA, AstraZeneca) [71], and GW654652 (GlaxoSmithKline) has been demonstrated in preclinical models with promising results in clinical trials of solid tumors. In MM, a phase II study of SU5416 in patients with refractory MM had only minimal clinical activity. However, signs of biological activity (decrease in plasma VEGF levels) suggested that angiogenic modulation may be of therapeutic value in patients with MM [72]. Similarly results were obtained in a targeted phase I/II trial of ZD6474 in relapsed or refractory MM [73].

15.5 Additional Approaches Directly or Indirectly Targeting VEGF

15.5.1 Thalidomide (alpha-N{phthalimido}glutarimide)/IMiDs

In 1957 thalidomide was introduced as a sedative and for treatment of morning sickness during the first trimester of pregnancy in Europe but got withdrawn from the market only a few years later due to the induction of teratogenicity and phocomelia. Based upon both its antiangiogenic activity [10] and the increased MVD in advanced MM [4], Singhal et al. used thalidomide to empirically treat patients with refractory or relapsed MM. Response rates of approximately one third of patients [11] were later confirmed by other investigators [74]. Today, thalidomide is part of standard therapy regimens not only for relapsed and refractory MM patients but also for treatment of newly diagnosed patients both transplant-eligible and transplant-ineligible. Importantly, thalidomide is also used as a category 1 maintenance therapeutic.

Subsequently to thalidomide, a series of more potent immunomodulatory drugs (IMiDs) have been developed [75]. Similar to thalidomide, IMiDs, and lenalidomide in particular are now part of standard therapy regimens for patients with relapsed or refractory MM, as well as for patients with newly diagnosed MM [76]. Moreover, recent data support the use of maintenance lenalidomide to prolong PFS post-ASCT [77, 78]. In addition, Palumbo et al. have shown prolonged PFS in non-ASCT candidates treated with melphalan plus prednisone plus lenalidomide followed by lenalidomide maintenance [79]. Importantly, both thalidomide and the IMiDs can overcome the growth and survival advantage conferred by the BM milieu, at least in part by downregulating VEGF [80, 81]. Mechanistically, recent data show that thalidomide significantly inhibits proliferation and capillarogenesis of MMECs [32] and mediates its antiangiogenic action by ceramide through depletion of VEGF receptors and antagonization by sphingosine-1-phosphate [82]. A phase I/II study using IMiD CC-4047 (Actimid), another IMiD in patients with advanced MM, showed anti-tumor activity and an acceptable safety profile [83].

15.5.2 Bortezomib (Velcade®)

The first-in-class proteasome inhibitor bortezomib (Velcade, PS341) may serve as a paradigm for the translation of a rationally designed and preclinically evaluated compound into successful clinical trials [84]. Bortezomib significantly changed treatment strategies in MM. It is now part of standard therapy regimens not only for relapsed and refractory MM patients but also for treatment of newly diagnosed patients both transplant-eligible and transplant-ineligible. Moreover, clinical trials to evaluate its role in maintenance therapy are ongoing.

The antiangiogenic effect of bortezomib [85, 86], which is mediated via caveolin-1-dependent regulation of MM cell-derived VEGF secretion and caveolin-1-mediated HUVEC proliferation, at least in part, additionally contributes to its anti-MM activity [87, 88].

15.5.3 CD40 Antibody

CD40 activation induces p53-dependent VEGF secretion [89]; conversely, a humanized anti-CD40 antibody induces cytotoxicity in human MM cells [90]. A derived clinical trial is ongoing.

15.5.4 Enzastaurin (LY317615.HCl)

The macrocyclic bisindolylmaleimide Enzastaurin (LY317615.HCl) is a novel orally available PKC inhibitor. Enzastaurin competes with ATP for the nucleotide

triphosphate-binding site of PKC, thereby blocking its activation. Besides its major target PKCβ, Enzastaurin also potently inhibits other PKC isoforms including PKCδ, PKCε, PKCγ, and PKCα [91]. Initially developed as an anti-angiogenic cancer therapy which both inhibits VEGF-triggered PKC pathways in endothelial cells as well as decreasing plasma VEGF levels [92–94], significant activity was also reported in a variety of tumor cells [91]. Early clinical studies show that Enzastaurin is well tolerated within a dosage range of 20–700 mg/day, without reaching a maximally tolerated dose, and mean steady-state plasma levels of 2 μM were achieved after oral administration of 525 mg/day Enzastaurin. In MM, PKC isoform expression has been reported in several MM cell lines and associated with MM cell apoptosis, migration, and IL-6 receptor α shedding.

Our own studies demonstrate marked in vitro and in vivo activity of Enzastaurin in MM via both its anti-tumor cell and antiangiogenic activity [95]. A study testing Enzastaurin in pretreated patients with Waldenstrom's macroglobulinemia or MM is ongoing. Similarly, several clinical trials are ongoing to test Enzastaurin in a variety of malignancies including recurrent brain tumor (phase I), advanced or metastatic malignancies (phase II), advanced non-small cell lung cancer (NSCLC) (phase II), metastatic colorectal cancer (phase II), advanced or metastatic pancreatic cancer combined with gemcitabine (phase II), glioblastoma multiforme (phase III), and diffuse large B-cell lymphoma (phase III) (http://www.clinicaltrials.gov).

15.6 Inhibiting Endothelial-Specific Integrin/Survival Signaling

The cyclic pentapeptide EMD 121974 (cilengitide) mediates its antiangiogenic activity via selective inhibition of integrins αvβ3 and αvβ5, which are co-stimulators in VEGF-triggered signaling pathways [96–99]. A clinical trial evaluating the efficacy of cilengitide in patients with locally advanced or metastatic cancer including MM has now been completed; results are pending.

15.7 Discussion

BM angiogenesis is pivotal for MM pathogenesis and therefore represents a potential therapeutic target in MM treatment. Indeed, the use of thalidomide and its derivative lenalidomide but also of bortezomib fundamentally changed treatment strategies in MM. The anti-MM activity of these agents is, at least in part, mediated via their anti-angiogenic effect. In addition, promising preclinical results obtained with more specific inhibitors of angiogenesis, e.g., VEGF-inhibitors, are now evaluated in the clinical setting.

Although the introduction of antiangiogenic agents into clinical practice represented a milestone event in cancer therapy during the last decade, the use of VEGF

inhibitors *alone* either in early or *refractory/progressive* disease was disappointing. Moreover, survival benefits observed in patients with *advanced* tumors treated with bevacizumab *even when combined* with conventional chemotherapies is modest, though significant [100, 101]. Therefore, further studies to increase our understanding of tumor angiogenesis and the development of resistance are required.

The optimal use of antiangiogenics/VEGF-inhibitors is very likely stage-dependent with higher sensitivity to VEGF inhibition in early stages due to the increase of redundant pro-angiogenic signals in later diseases [42]. Moreover, mechanisms mediating the potentiation of combination therapy likely dependent on timing and sequencing of the combined agents. This may explain why the clinical activity of VEGF-inhibitors as single agents has usually been disappointing when used in solid tumors. Even when given in combination with conventional chemotherapies, the activity of VEGF-inhibitors is only transient. In addition to upregulation of alternative pro-angiogenic signaling pathways, potential mechanisms of evasive resistance include the recruitment of vascular progenitor cells and pro-angiogenic monocytes from the bone marrow, increased and tight pericyte coverage, or increased capabilities for invasion and metastasis; pre-existing inflammatory cell-mediated vascular protection; hypovascularity; invasive and metastatic co-option of normal vessels; and mutational alteration of genes within endothelial cells [39, 102].

Therapeutic benefits may therefore be achieved by initiating treatment with VEGF-inhibitors early and by using antiangiogenic cocktails, which not only target VEGF both in patients with early and late stage disease. Moreover, new combined treatment regimens are attempting to reduce drug-associated toxic side effects and the development of drug resistance by either metronomic chemotherapy [102–104] or combination with other antiangiogenic agents [105–109]. Novel agents directed to overcome mechanisms of resistance against antiangiogenic therapies include vascular-disrupting agents (VDAs), which selectively destroy existing tumor vessels, and drugs, which target molecular mechanisms leading to vessel branching. Ongoing clinical trials study the activity of VDAs including combrestatin, AVE8062 DMXAA, TZT-1027, ZD6126, Exherin, MN-029 alone or in combination with conventional chemotherapies or radiotherapy. The addition of VDAs to antiangiogenic therapies may have a synergistic effect [110]. Additional novel approaches to inhibit tumor angiogenesis include third-generation antiangiogenic therapies targeting Dll4/Notch-, VEGFR-3-, PlGF and PDGF-, and maybe most promising Ang-TIE2-induced signaling cascades, as well as Hif-1 [111, 112]. Specifically, Ang-1 expression is up-regulated in MM cell lines or in plasma cells obtained from MM patients and correlates with BM MVD. Indeed, anti-Tie-2 antibodies blocked the in vitro angiogenic activity of MM cells [113].

Given the benefits of combination therapy, it is also crucial to optimize existing or identify new treatment regimens in order to reduce drug-associated toxic side effects. Anti-VEGF inhibitors are generally well tolerated. Surprisingly, a very common side effect of antiangiogenic therapy is hypertension which has been associated with NO changes, pruning of normal vessels, as well as effects on renal salt homeostasis [114]. Although hypertension is treatment-responsive, it remains to be determined whether hypertension inflicts a “contra-effect” to antiangiogenic/VEGF-inhibitor therapy.

Adverse side effects of direct VEGF-inhibition (bevacizumab, sorafenib, sutent) rarely also include neuropathy, although with a much lower incidence than bortezomib or thalidomide. A pivotal role of VEGF and other angiogenic molecules in neurogenesis and neuroprotection has been proposed [115]. In MM, thalidomide, bortezomib, and lenalidomide have fundamentally changed clinical practice. However, toxic peripheral neuropathy represents a dose-limiting debilitating side effect with high incidence and lack of effective treatment.

In summary, antiangiogenic compounds like thalidomide have already demonstrated activity in MM. However, with the increase of our knowledge of the complexity of molecular mechanisms contributing to tumor angiogenesis in general, and MM BM angiogenesis in particular, we aim to identify additional therapeutic targets to further optimize treatment regimens and to reduce mechanisms leading to antiangiogenic drug-resistance in order to further improve MM patient outcome and reduce drug toxicity.

References

1. Folkman J (1971) Tumor angiogenesis: therapeutic implications. N Engl J Med 285:1182–1186
2. Ferrara N (2004) Vascular endothelial growth factor as a target for anticancer therapy. Oncologist 9(Suppl 1):2–10
3. Podar K, Richardson PG, Chauhan D, Anderson KC (2007) Targeting the vascular endothelial growth factor pathway in the treatment of multiple myeloma. Expert Rev Anticancer Ther 7:551–566
4. Vacca A, Ribatti D, Roncali L, Ranieri G, Serio G, Silvestris F, Dammacco F (1994) Bone marrow angiogenesis and progression in multiple myeloma. Br J Haematol 87:503–508
5. Sezer O, Niemoller K, Eucker J, Jakob C, Kaufmann O, Zavrski I, Dietel M, Possinger K (2000) Bone marrow microvessel density is a prognostic factor for survival in patients with multiple myeloma. Ann Hematol 79:574–577
6. Rajkumar SV, Leong T, Roche PC, Fonseca R, Dispenzieri A, Lacy MQ, Lust JA, Witzig TE, Kyle RA, Gertz MA, Greipp PR (2000) Prognostic value of bone marrow angiogenesis in multiple myeloma. Clin Cancer Res 6:3111–3116
7. Kumar S, Fonseca R, Dispenzieri A, Lacy MQ, Lust JA, Wellik L, Witzig TE, Gertz MA, Kyle RA, Greipp PR, Rajkumar SV (2003) Prognostic value of angiogenesis in solitary bone plasmacytoma. Blood 101:1715–1717
8. Kumar S, Litzow MR, Rajkumar SV (2003) Effect of allogeneic stem cell transplantation on bone marrow angiogenesis in chronic myelogenous leukemia. Bone Marrow Transplant 32:1065–1069
9. Oh HS, Choi JH, Park CK, Jung CW, Lee SI, Park Q, Suh C, Kim SB, Chi HS, Lee JH, Cho EK, Bang SM, Ahn MJ (2002) Comparison of microvessel density before and after peripheral blood stem cell transplantation in multiple myeloma patients and its clinical implications: multicenter trial. Int J Hematol 76:465–470
10. D'Amato RJ, Loughnan MS, Flynn E, Folkman J (1994) Thalidomide is an inhibitor of angiogenesis. Proc Natl Acad Sci USA 91:4082–4085
11. Singhal S, Mehta J, Desikan R, Ayers D, Roberson P, Eddlemon P, Munshi N, Anaissie E, Wilson C, Dhodapkar M, Zeddis J, Barlogie B (1999) Antitumor activity of thalidomide in refractory multiple myeloma. N Engl J Med 341:1565–1571
12. Kumar S, Gertz MA, Dispenzieri A, Lacy MQ, Wellik LA, Fonseca R, Lust JA, Witzig TE, Kyle RA, Greipp PR, Rajkumar SV (2004) Prognostic value of bone marrow angiogenesis in

patients with multiple myeloma undergoing high-dose therapy. Bone Marrow Transplant 34(3):235–239
13. Bhatti SS, Kumar L, Dinda AK, Dawar R (2006) Prognostic value of bone marrow angiogenesis in multiple myeloma: use of light microscopy as well as computerized image analyzer in the assessment of microvessel density and total vascular area in multiple myeloma and its correlation with various clinical, histological, and laboratory parameters. Am J Hematol 81:649–656
14. Vacca A, Ria R, Ribatti D, Semeraro F, Djonov V, Di Raimondo F, Dammacco F (2003) A paracrine loop in the vascular endothelial growth factor pathway triggers tumor angiogenesis and growth in multiple myeloma. Haematologica 88:176–185
15. Dankbar B, Padro T, Leo R, Feldmann B, Kropff M, Mesters RM, Serve H, Berdel WE, Kienast J (2000) Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interactions in multiple myeloma. Blood 95:2630–2636
16. Vacca A, Ribatti D, Presta M, Minischetti M, Iurlaro M, Ria R, Albini A, Bussolino F, Dammacco F (1999) Bone marrow neovascularization, plasma cell angiogenic potential, and matrix metalloproteinase-2 secretion parallel progression of human multiple myeloma. Blood 93:3064–3073
17. Bisping G, Leo R, Wenning D, Dankbar B, Padro T, Kropff M, Scheffold C, Kroger M, Mesters RM, Berdel WE, Kienast J (2003) Paracrine interactions of basic fibroblast growth factor and interleukin-6 in multiple myeloma. Blood 101:2775–2783
18. Borset M, Hjorth-Hansen H, Seidel C, Sundan A, Waage A (1996) Hepatocyte growth factor and its receptor c-met in multiple myeloma. Blood 88:3998–4004
19. Podar K, Chauhan D, Anderson KC (2009) Bone marrow microenvironment and the identification of new targets for myeloma therapy. Leukemia 23:10–24
20. Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ (1999) Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science 284:1994–1998
21. Rafii S, Lyden D, Benezra R, Hattori K, Heissig B (2002) Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? Nat Rev Cancer 2:826–835
22. Folberg R, Hendrix MJ, Maniotis AJ (2000) Vasculogenic mimicry and tumor angiogenesis. Am J Pathol 156:361–381
23. Chang YS, di Tomaso E, McDonald DM, Jones R, Jain RK, Munn LL (2000) Mosaic blood vessels in tumors: frequency of cancer cells in contact with flowing blood. Proc Natl Acad Sci USA 97:14608–14613
24. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J (2000) Vascular-specific growth factors and blood vessel formation. Nature 407:242–248
25. Hendrix MJ, Seftor EA, Meltzer PS, Gardner LM, Hess AR, Kirschmann DA, Schatteman GC, Seftor RE (2001) Expression and functional significance of VE-cadherin in aggressive human melanoma cells: role in vasculogenic mimicry. Proc Natl Acad Sci USA 98:8018–8023
26. Folkman J (2001) Can mosaic tumor vessels facilitate molecular diagnosis of cancer? Proc Natl Acad Sci USA 98:398–400
27. Rafii S (2000) Circulating endothelial precursors: mystery, reality, and promise. J Clin Invest 105:17–19
28. Dvorak HF, Nagy JA, Dvorak JT, Dvorak AM (1988) Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. Am J Pathol 133:95–109
29. Morikawa S, Baluk P, Kaidoh T, Haskell A, Jain RK, McDonald DM (2002) Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. Am J Pathol 160:985–1000
30. Baish JW, Jain RK (2000) Fractals and cancer. Cancer Res 60:3683–3688
31. Helmlinger G, Yuan F, Dellian M, Jain RK (1997) Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. Nat Med 3:177–182
32. Vacca A, Ria R, Semeraro F, Merchionne F, Coluccia M, Boccarelli A, Scavelli C, Nico B, Gernone A, Battelli F, Tabilio A, Guidolin D, Petrucci MT, Ribatti D, Dammacco F (2003) Endothelial cells in the bone marrow of patients with multiple myeloma. Blood 102:3340–3348

33. Gupta D, Treon SP, Shima Y, Hideshima T, Podar K, Tai YT, Lin B, Lentzsch S, Davies FE, Chauhan D, Schlossman RL, Richardson P, Ralph P, Wu L, Payvandi F, Muller G, Stirling DI, Anderson KC (2001) Adherence of multiple myeloma cells to bone marrow stromal cells upregulates vascular endothelial growth factor secretion: therapeutic applications. Leukemia 15:1950–1961
34. Urashima M, Chauhan D, Hatziyanni M, Ogata A, Hollenbaugh D, Aruffo A, Anderson KC (1996) CD40 ligand triggers interleukin-6 mediated B cell differentiation. Leuk Res 20:507–515
35. Tai YT, Podar K, Mitsiades N, Lin B, Mitsiades C, Gupta D, Akiyama M, Catley L, Hideshima T, Munshi NC, Treon SP, Anderson KC (2003) CD40 induces human multiple myeloma cell migration via phosphatidylinositol 3-kinase/AKT/NF-kappa B signaling. Blood 101:2762–2769
36. Gagne P, Akalu A, Brooks PC (2004) Challenges facing antiangiogenic therapy for cancer: impact of the tumor extracellular environment. Expert Rev Anticancer Ther 4:129–140
37. Ribatti D, Nico B, Vacca A (2006) Importance of the bone marrow microenvironment in inducing the angiogenic response in multiple myeloma. Oncogene 25:4257–4266
38. Kerbel R, Folkman J (2002) Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2:727–739
39. Bergers G, Benjamin LE (2003) Tumorigenesis and the angiogenic switch. Nat Rev Cancer 3:401–410
40. Podar K, Anderson KC (2005) The pathophysiological role of VEGF in hematological malignancies: therapeutic implications. Blood 105:1383–1395
41. Yaccoby S, Barlogie B, Epstein J (1998) Primary myeloma cells growing in SCID-hu mice: a model for studying the biology and treatment of myeloma and its manifestations. Blood 92:2908–2913
42. Casanovas O, Hicklin DJ, Bergers G, Hanahan D (2005) Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer Cell 8:299–309
43. Willett CG, Duda DG, di Tomaso E, Boucher Y, Ancukiewicz M, Sahani DV, Lahdenranta J, Chung DC, Fischman AJ, Lauwers GY, Shellito P, Czito BG, Wong TZ, Paulson E, Poleski M, Vujaskovic Z, Bentley R, Chen HX, Clark JW, Jain RK (2009) Efficacy, safety, and biomarkers of neoadjuvant bevacizumab, radiation therapy, and fluorouracil in rectal cancer: a multidisciplinary phase II study. J Clin Oncol 27:3020–3026
44. Ferrara N, Hillan KJ, Gerber HP, Novotny W (2004) Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov 3:391–400
45. Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, Ioffe E, Huang T, Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yancopoulos GD, Rudge JS (2002) VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci USA 99:11393–11398
46. Chu QS (2009) Aflibercept (AVE0005): an alternative strategy for inhibiting tumour angiogenesis by vascular endothelial growth factors. Expert Opin Biol Ther 9:263–271
47. Kim ES, Serur A, Huang J, Manley CA, McCrudden KW, Frischer JS, Soffer SZ, Ring L, New T, Zabski S, Rudge JS, Holash J, Yancopoulos GD, Kandel JJ, Yamashiro DJ (2002) Potent VEGF blockade causes regression of coopted vessels in a model of neuroblastoma. Proc Natl Acad Sci USA 99:11399–11404
48. Jayson GC, Zweit J, Jackson A, Mulatero C, Julyan P, Ranson M, Broughton L, Wagstaff J, Hakannson L, Groenewegen G, Bailey J, Smith N, Hastings D, Lawrance J, Haroon H, Ward T, McGown AT, Tang M, Levitt D, Marreaud S, Lehmann FF, Herold M, Zwierzina H (2002) Molecular imaging and biological evaluation of HuMV833 anti-VEGF antibody: implications for trial design of antiangiogenic antibodies. J Natl Cancer Inst 94:1484–1493
49. Jayson GC, Parker GJ, Mullamitha S, Valle JW, Saunders M, Broughton L, Lawrance J, Carrington B, Roberts C, Issa B, Buckley DL, Cheung S, Davies K, Watson Y, Zinkewich-Peotti K, Rolfe L, Jackson A (2005) Blockade of platelet-derived growth factor receptor-beta by CDP860, a humanized, PEGylated di-Fab', leads to fluid accumulation and is associated with increased tumor vascularized volume. J Clin Oncol 23:973–981

50. Lowinger TB, Riedl B, Dumas J, Smith RA (2002) Design and discovery of small molecules targeting raf-1 kinase. Curr Pharm Des 8:2269–2278
51. Richly H, Kupsch P, Passage K, Grubert M, Hilger RA, Kredtke S, Voliotis D, Scheulen ME, Seeber S, Strumberg D (2003) A phase I clinical and pharmacokinetic study of the Raf kinase inhibitor (RKI) BAY 43–9006 administered in combination with doxorubicin in patients with solid tumors. Int J Clin Pharmacol Ther 41:620–621
52. O'Farrell AM, Abrams TJ, Yuen HA, Ngai TJ, Louie SG, Yee KW, Wong LM, Hong W, Lee LB, Town A, Smolich BD, Manning WC, Murray LJ, Heinrich MC, Cherrington JM (2003) SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity in vitro and in vivo. Blood 101:3597–3605
53. Motzer RJ, Hudes GR, Curti BD, McDermott DF, Escudier BJ, Negrier S, Duclos B, Moore L, O'Toole T, Boni JP, Dutcher JP (2007) Phase I/II trial of temsirolimus combined with interferon alfa for advanced renal cell carcinoma. J Clin Oncol 25:3958–3964
54. Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Oudard S, Negrier S, Szczylik C, Pili R, Bjarnason GA, Garcia-del-Muro X, Sosman JA, Solska E, Wilding G, Thompson JA, Kim ST, Chen I, Huang X, Figlin RA (2009) Overall survival and updated results for sunitinib compared with interferon alfa in patients with metastatic renal cell carcinoma. J Clin Oncol 27:3584–3590
55. George S, Merriam P, Maki RG, Van den Abbeele AD, Yap JT, Akhurst T, Harmon DC, Bhuchar G, O'Mara MM, D'Adamo DR, Morgan J, Schwartz GK, Wagner AJ, Butrynski JE, Demetri GD, Keohan ML (2009) Multicenter phase II trial of sunitinib in the treatment of nongastrointestinal stromal tumor sarcomas. J Clin Oncol 27:3154–3160
56. Ramakrishnan V, Timm M, Haug JL, Kimlinger TK, Wellik LE, Witzig TE, Rajkumar SV, Adjei AA, Kumar S (2010) Sorafenib, a dual Raf kinase/vascular endothelial growth factor receptor inhibitor has significant anti-myeloma activity and synergizes with common anti-myeloma drugs. Oncogene 29:1190–1202
57. Wood JM, Bold G, Buchdunger E, Cozens R, Ferrari S, Frei J, Hofmann F, Mestan J, Mett H, O'Reilly T, Persohn E, Rosel J, Schnell C, Stover D, Theuer A, Towbin H, Wenger F, Woods-Cook K, Menrad A, Siemeister G, Schirner M, Thierauch KH, Schneider MR, Drevs J, Martiny-Baron G, Totzke F (2000) PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. Cancer Res 60: 2178–2189
58. Thomas AL, Morgan B, Drevs J, Unger C, Wiedenmann B, Vanhoefer U, Laurent D, Dugan M, Steward WP (2003) Vascular endothelial growth factor receptor tyrosine kinase inhibitors: PTK787/ZK 222584. Semin Oncol 30:32–38
59. Lin B, Podar K, Gupta D, Tai YT, Li S, Weller E, Hideshima T, Lentzsch S, Davies F, Li C, Weisberg E, Schlossman RL, Richardson PG, Griffin JD, Wood J, Munshi NC, Anderson KC (2002) The vascular endothelial growth factor receptor tyrosine kinase inhibitor PTK787/ZK222584 inhibits growth and migration of multiple myeloma cells in the bone marrow microenvironment. Cancer Res 62:5019–5026
60. Vij R, Ansstas G, Mosley JC, Bryant G, Hassan A, Amador-Ortiz C, Procknow E (2010) Efficacy and tolerability of PTK787/ZK 222584 in a phase II study of post-transplant maintenance therapy in patients with multiple myeloma following high-dose chemotherapy and autologous stem cell transplant. Leuk Lymphoma 51:1577–1579
61. Kumar R, Knick VB, Rudolph SK, Johnson JH, Crosby RM, Hopper TM, Miller CG, Harrington LE, Onori JA, Mullin RJ, Gilmer TM, Truesdale AT, Epperly AH, Bolor A, Cheung M, Stafford JA, Luttrell DK (2005). GW786034: a pan-inhibitor of VEGF receptors with potent anti-tumor and anti-angiogenic activity. In: AACR-NCI-EORTC international conference – molecular targets and cancer therapeutics, pp 58–59
62. GlaxoSmithKline Pazopanib Hydrochloride (2006) Pharmacop Forum 32*:* 217
63. Hurwitz HI, Dowlati A, Saini S, Savage S, Suttle AB, Gibson DM, Hodge JP, Merkle EM, Pandite L (2009) Phase I trial of pazopanib in patients with advanced cancer. Clin Cancer Res 15:4220–4227

64. Sternberg CN, Davis ID, Mardiak J, Szczylik C, Lee E, Wagstaff J, Barrios CH, Salman P, Gladkov OA, Kavina A, Zarba JJ, Chen M, McCann L, Pandite L, Roychowdhury DF, Hawkins RE (2010) Pazopanib in locally advanced or metastatic renal cell carcinoma: results of a randomized phase III trial. J Clin Oncol 28:1061–1068
65. Podar K, Tonon G, Sattler M, Tai YT, Legouill S, Yasui H, Ishitsuka K, Kumar S, Kumar R, Pandite LN, Hideshima T, Chauhan D, Anderson KC (2006) The small-molecule VEGF receptor inhibitor pazopanib (GW786034B) targets both tumor and endothelial cells in multiple myeloma. Proc Natl Acad Sci USA 103(51):19478–19483
66. Prince HM, Honemann D, Spencer A, Rizzieri DA, Stadtmauer EA, Roberts AW, Bahlis N, Tricot G, Bell B, Demarini DJ, Benjamin Suttle A, Baker KL, Pandite LN (2009) Vascular endothelial growth factor inhibition is not an effective therapeutic strategy for relapsed or refractory multiple myeloma: a phase 2 study of pazopanib (GW786034). Blood 113: 4819–4820
67. O'Farrell AM, Yuen HA, Smolich B, Hannah AL, Louie SG, Hong W, Stopeck AT, Silverman LR, Lancet JE, Karp JE, Albitar M, Cherrington JM, Giles FJ (2004) Effects of SU5416, a small molecule tyrosine kinase receptor inhibitor, on FLT3 expression and phosphorylation in patients with refractory acute myeloid leukemia. Leuk Res 28:679–689
68. Giles FJ, Stopeck AT, Silverman LR, Lancet JE, Cooper MA, Hannah AL, Cherrington JM, O'Farrell AM, Yuen HA, Louie SG, Hong W, Cortes JE, Verstovsek S, Albitar M, O'Brien SM, Kantarjian HM, Karp JE (2003) SU5416, a small molecule tyrosine kinase receptor inhibitor, has biologic activity in patients with refractory acute myeloid leukemia or myelodysplastic syndromes. Blood 102:795–801
69. Fiedler W, Mesters R, Tinnefeld H, Loges S, Staib P, Duhrsen U, Flasshove M, Ottmann OG, Jung W, Cavalli F, Kuse R, Thomalla J, Serve H, O'Farrell AM, Jacobs M, Brega NM, Scigalla P, Hossfeld DK, Berdel WE (2003) A phase 2 clinical study of SU5416 in patients with refractory acute myeloid leukemia. Blood 102:2763–2767
70. Beebe JS, Jani JP, Knauth E, Goodwin P, Higdon C, Rossi AM, Emerson E, Finkelstein M, Floyd E, Harriman S, Atherton J, Hillerman S, Soderstrom C, Kou K, Gant T, Noe MC, Foster B, Rastinejad F, Marx MA, Schaeffer T, Whalen PM, Roberts WG (2003) Pharmacological characterization of CP-547,632, a novel vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for cancer therapy. Cancer Res 63:7301–7309
71. Bates D (2003) ZD-6474 AstraZeneca. Curr Opin Investig Drugs 4:1468–1472
72. Zangari M, Anaissie E, Stopeck A, Morimoto A, Tan N, Lancet J, Cooper M, Hannah A, Garcia-Manero G, Faderl S, Kantarjian H, Cherrington J, Albitar M, Giles FJ (2004) Phase II study of SU5416, a small molecule vascular endothelial growth factor tyrosine kinase receptor inhibitor, in patients with refractory multiple myeloma. Clin Cancer Res 10:88–95
73. Morabito A, Piccirillo MC, Falasconi F, De Feo G, Del Giudice A, Bryce J, Di Maio M, De Maio E, Normanno N, Perrone F (2009) Vandetanib (ZD6474), a dual inhibitor of vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptor (EGFR) tyrosine kinases: current status and future directions. Oncologist 14:378–390
74. Mazumder A, Jagannath S (2006) Thalidomide and lenalidomide in multiple myeloma. Best Pract Res Clin Haematol 19:769–780
75. Bartlett JB, Dredge K, Dalgleish AG (2004) The evolution of thalidomide and its IMiD derivatives as anticancer agents. Nat Rev Cancer 4:314–322
76. Richardson P, Mitsiades C, Laubach J, Schlossman R, Ghobrial I, Hideshima T, Munshi N, Anderson K (2010) Lenalidomide in multiple myeloma: an evidence-based review of its role in therapy. Core Evid 4:215–245
77. Attal M, Cristini C, Marit G, Caillot D, Facon T, Hullin C, Moreau P, Mathiot C, Avet-Loiseau H, Harousseau J, Myelome I.F.d (2010) Lenalidomide maintenance after transplantation for myeloma. J Clin Oncol 28: 15s (suppl; abstr. 8018)
78. McCarthy P, Owzar K, Anderson K, Hofmeister C, Hassoun H, Hurd D, Stadtmauer E, Giralt S, Hars V, Linker C, CALGB E.a.B.-C (2010) Phase III intergroup study of lenalidomide versus placebo maintenance therapy following single autologous stem cell transplant (ASCT) for multiple myeloma (MM): CALGB 100104. Clin Oncol 28: 15s (suppl; abstr 8017)

79. Palumbo A, Falco P, Benevolo G et al. (2010) A multicenter, open label study of oral lenalidomide and prednisone (RP) followed by oral lenalidomide, melphalan and prednisone (MPR) and oral lenalidomide maintenance in newly diagnosed elderly multiple myeloma patients. Blood 116:1940. abstract
80. Hideshima T, Chauhan D, Shima Y, Raje N, Davies FE, Tai YT, Treon SP, Lin B, Schlossman RL, Richardson P, Muller G, Stirling DI, Anderson KC (2000) Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy. Blood 96:2943–2950
81. D'Amato RJ, Lentzsch S, Anderson KC, Rogers MS (2001) Mechanism of action of thalidomide and 3-aminothalidomide in multiple myeloma. Semin Oncol 28:597–601
82. Yabu T, Tomimoto H, Taguchi Y, Yamaoka S, Igarashi Y, Okazaki T (2005) Thalidomide-induced anti-angiogenic action is mediated by ceramide through depletion of VEGF receptors, and antagonized by sphingosine-1-phosphate. Blood 106(1):125–134
83. Schey SA, Fields P, Bartlett JB, Clarke IA, Ashan G, Knight RD, Streetly M, Dalgleish AG (2004) Phase I study of an immunomodulatory thalidomide analog, CC-4047, in relapsed or refractory multiple myeloma. J Clin Oncol 22:3269–3276
84. Kane RC, Bross PF, Farrell AT, Pazdur R (2003) Velcade: U.S. FDA approval for the treatment of multiple myeloma progressing on prior therapy. Oncologist 8:508–513
85. Nawrocki ST, Bruns CJ, Harbison MT, Bold RJ, Gotsch BS, Abbruzzese JL, Elliott P, Adams J, McConkey DJ (2002) Effects of the proteasome inhibitor PS-341 on apoptosis and angiogenesis in orthotopic human pancreatic tumor xenografts. Mol Cancer Ther 1:1243–1253
86. Oikawa T, Sasaki T, Nakamura M, Shimamura M, Tanahashi N, Omura S, Tanaka K (1998) The proteasome is involved in angiogenesis. Biochem Biophys Res Commun 246:243–248
87. LeBlanc R, Catley LP, Hideshima T, Lentzsch S, Mitsiades CS, Mitsiades N, Neuberg D, Goloubeva O, Pien CS, Adams J, Gupta D, Richardson PG, Munshi NC, Anderson KC (2002) Proteasome inhibitor PS-341 inhibits human myeloma cell growth in vivo and prolongs survival in a murine model. Cancer Res 62:4996–5000
88. Podar K, Shringarpure R, Tai YT, Simoncini M, Sattler M, Ishitsuka K, Richardson PG, Hideshima T, Chauhan D, Anderson KC (2004) Caveolin-1 is required for vascular endothelial growth factor-triggered multiple myeloma cell migration and is targeted by bortezomib. Cancer Res 64:7500–7506
89. Tai YT, Podar K, Gupta D, Lin B, Young G, Akiyama M, Anderson KC (2002) CD40 activation induces p53-dependent vascular endothelial growth factor secretion in human multiple myeloma cells. Blood 99:1419–1427
90. Tai YT, Catley LP, Mitsiades CS, Burger R, Podar K, Shringpaure R, Hideshima T, Chauhan D, Hamasaki M, Ishitsuka K, Richardson P, Treon SP, Munshi NC, Anderson KC (2004) Mechanisms by which SGN-40, a humanized anti-CD40 antibody, induces cytotoxicity in human multiple myeloma cells: clinical implications. Cancer Res 64:2846–2852
91. Graff JR, McNulty AM, Hanna KR, Konicek BW, Lynch RL, Bailey SN, Banks C, Capen A, Goode R, Lewis JE, Sams L, Huss KL, Campbell RM, Iversen PW, Neubauer BL, Brown TJ, Musib L, Geeganage S, Thornton D (2005) The protein kinase Cbeta-selective inhibitor, Enzastaurin (LY317615.HCl), suppresses signaling through the AKT pathway, induces apoptosis, and suppresses growth of human colon cancer and glioblastoma xenografts. Cancer Res 65:7462–7469
92. Keyes K, Cox K, Treadway P, Mann L, Shih C, Faul MM, Teicher BA (2002) An in vitro tumor model: analysis of angiogenic factor expression after chemotherapy. Cancer Res 62:5597–5602
93. Keyes KA, Mann L, Sherman M, Galbreath E, Schirtzinger L, Ballard D, Chen YF, Iversen P, Teicher BA (2004) LY317615 decreases plasma VEGF levels in human tumor xenograft-bearing mice. Cancer Chemother Pharmacol 53:133–140
94. Herbst RS (2002) Targeted therapy using novel agents in the treatment of non-small-cell lung cancer. Clin Lung Cancer 3(Suppl 1):S30–S38
95. Podar K, Raab MS, Zhang J, McMillin D, Breitkreutz I, Tai YT, Lin BK, Munshi N, Hideshima T, Chauhan D, Anderson KC (2007) Targeting PKC in multiple myeloma: in vitro and in vivo

effects of the novel, orally available small-molecule inhibitor enzastaurin (LY317615.HCl). Blood 109:1669–1677
96. MacDonald TJ, Taga T, Shimada H, Tabrizi P, Zlokovic BV, Cheresh DA, Laug WE (2001) Preferential susceptibility of brain tumors to the antiangiogenic effects of an alpha(v) integrin antagonist. Neurosurgery 48:151–157
97. Burke PA, DeNardo SJ, Miers LA, Lamborn KR, Matzku S, DeNardo GL (2002) Cilengitide targeting of alpha(v)beta(3) integrin receptor synergizes with radioimmunotherapy to increase efficacy and apoptosis in breast cancer xenografts. Cancer Res 62:4263–4272
98. Raguse JD, Gath HJ, Bier J, Riess H, Oettle H (2004) Cilengitide (EMD 121974) arrests the growth of a heavily pretreated highly vascularised head and neck tumour. Oral Oncol 40:228–230
99. Nisato RE, Tille JC, Jonczyk A, Goodman SL, Pepper MS (2003) alphav beta 3 and alphav beta 5 integrin antagonists inhibit angiogenesis in vitro. Angiogenesis 6:105–119
100. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350:2335–2342
101. Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, Lilenbaum R, Johnson DH (2006) Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med 355:2542–2550
102. Kerbel RS (2006) Antiangiogenic therapy: a universal chemosensitization strategy for cancer? Science 312:1171–1175
103. Hanahan D, Bergers G, Bergsland E (2000) Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. J Clin Invest 105:1045–1047
104. Kerbel RS, Kamen BA (2004) The anti-angiogenic basis of metronomic chemotherapy. Nat Rev Cancer 4:423–436
105. Browder T, Butterfield CE, Kraling BM, Shi B, Marshall B, O'Reilly MS, Folkman J (2000) Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res 60:1878–1886
106. Gasparini G (2001) Metronomic scheduling: the future of chemotherapy? Lancet Oncol 2:733–740
107. Kamen BA, Rubin E, Aisner J, Glatstein E (2000) High-time chemotherapy or high time for low dose. J Clin Oncol 18:2935–2937
108. Kerbel RS, Klement G, Pritchard KI, Kamen B (2002) Continuous low-dose anti-angiogenic/metronomic chemotherapy: from the research laboratory into the oncology clinic. Ann Oncol 13:12–15
109. Pietras K, Hanahan D (2005) A multitargeted, metronomic, and maximum-tolerated dose "chemo-switch" regimen is antiangiogenic, producing objective responses and survival benefit in a mouse model of cancer. J Clin Oncol 23:939–952
110. Klasa RJ, List AF, Cheson BD (2001) Rational approaches to design of therapeutics targeting molecular markers. Am Soc Hematol Educ Program 2001(1):443–462
111. Grothey A, Galanis E (2009) Targeting angiogenesis: progress with anti-VEGF treatment with large molecules. Nat Rev Clin Oncol 6:507–518
112. Ivy SP, Wick JY, Kaufman BM (2009) An overview of small-molecule inhibitors of VEGFR signaling. Nat Rev Clin Oncol 6:569–579
113. Giuliani N, Colla S, Lazzaretti M, Sala R, Roti G, Mancini C, Bonomini S, Lunghi P, Hojden M, Genestreti G, Svaldi M, Coser P, Fattori PP, Sammarelli G, Gazzola GC, Bataille R, Almici C, Caramatti C, Mangoni L, Rizzoli V (2003) Proangiogenic properties of human myeloma cells: production of angiopoietin-1 and its potential relationship to myeloma-induced angiogenesis. Blood 102:638–645
114. Carmeliet P (2005) Angiogenesis in life, disease and medicine. Nature 438:932–936
115. Greenberg DA, Jin K (2005) From angiogenesis to neuropathology. Nature 438:954–959

Chapter 16
Novel In Vivo Models in Myeloma

Eric Sanchez, Haiming Chen, and James R. Berenson

Abstract Biologically relevant and reproducible in vivo models are commonly used to evaluate new therapies and targets for the treatment of multiple myeloma (MM). This chapter describes the historical development of the transplantability of tissues from one species to that of a foreign or different species (hetero-transplantation), the development of non-human mouse models of plasmacytomas and myelomas, other new animal models of MM, and how the knowledge gained from human MM models has been applied to clinical trials directly benefiting MM patients.

16.1 Introduction

Animal models of human cancer have long ago been of scientific interest. James B. Murphy demonstrated in 1912 that a cancer of the rat, specifically a sarcoma, grew in the outer membrane (fused chorion and allantois) of chick embryos before hatching time [1]. Furthermore, the rat sarcoma could be serially transferred from embryo to embryo indefinitely and could also be implanted back into the rat after generations of growth in the chick embryo. His experiments in 1914 also showed that small round lymphocytes were responsible for the rejection of a rat sarcoma growing in the outer membrane of chick embryos [2]. The small round lymphocytes were derived from adult chicken spleen and bone marrow but adult thymus cells were not tested in these experiments. Thus, it was known that cells of at least two organs (spleen and bone marrow) were immunologically competent and able to reject tissue grafts. A few decades later, heterologous mammalian transplantation was demonstrated by successful growth and serial transfer of rabbit tumors into the anterior chamber of the eye of guinea pigs [3]. This inoculation method suggested that it might be

E. Sanchez • H. Chen • J.R. Berenson, M.D. (✉)
Institute for Myeloma and Bone Cancer Research,
9201 W. Sunset Blvd., Suite 300, West Hollywood, CA 90069, USA
e-mail: jberenson@imbcr.org

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8_16,

possible to grow human cancers. A preliminary report in 1938 by Harry S.N. Green briefly mentions the growth of a human scirrhus cancer of the breast using this inoculation method [4]; however, detailed experiments in 1950 established unequivocally the successful heterotransplantation of human melanomas into the anterior chamber of the eye of guinea pigs [5]. Serial transfer of these human melanomas into subsequent guinea pigs' eyes is not mentioned in the report. It is important to note that all of the above-mentioned studies were performed either in anatomical locations deprived of immune competent cells or immunologically privileged sites. Detailed literature dealing with heterologous transplantation of mammalian tumors is available by each of these and other authors, as various claims to be the first to successfully grow human cancers in foreign species have been made but were not substantiated upon critical scientific review.

In 1960, it was demonstrated that human cell lines could grow in rats if injected less than 24 h after birth [6] and additional experiments, by other authors, in 1966 and 1976 confirmed this observation [7, 8], respectively. The role of the thymus was unknown in the early 1960s. In humans, it was known that in infancy the thymus was a large mass of tissue that decreases in size at or following puberty and involutes in the adult. Prevailing opinion at the time was that the thymus was a graveyard for dying cells. A major discovery in immunology was made in 1962 when J.F.A.P. Miller demonstrated that mice thymectomized at birth, but not later, failed to reject skin from a different species, the rat [9]. The rationale for grafting mice with foreign skin grafts was based on his observation that neonatally thymectomized mice showed a marked deficiency of lymphocytes which was accompanied by an inordinate susceptibility to infection.

Shortly thereafter, in 1966, the mouse mutant "nude" mouse was described [10], and in 1968 it was found not to contain a thymus [11]. Homozygote nudes are hairless, lacking a thymus, and show extremely low blood leukocyte counts. The successful graft or "take" of a human cancer was demonstrated in 1969 [12]. Researchers now had a scientific tool to study the transplantability of foreign tissues (normal and cancerous), the factors of resistance governing heterologous tissue grafts and a model system to study human cancer chemotherapy without placing the patients at risk. In 1983, the severe combined immunodeficiency (SCID) mutation occurred spontaneously in the BALB/c C.B-17 strain [13]. SCID mice lack functional B and T cells and are therefore ideally suited for use as animal models of human cancers as they more readily accept transplantation of human tissues. This essentially means that these mice have a diminished immune system making it easier for the transplanted human MM to grow in the mouse.

A report in 1992 showed the first successful engraftment of primary human MM cells when injected intraperitonally into SCID mice [14]. Growth of human MM cell lines using human fetal bone implants was demonstrated in 1997 [15]. Irradiated SCID mice were implanted with bilateral human fetal bone grafts and human MM cell lines were injected only into the marrow cavity of the left bone implant. It was observed that MM cell lines engrafted and proliferated first in the left and then in the right human fetal bone implant. Additionally, these human MM cell lines were also found to spread to other locations in the SCID mice that did not have human fetal

bone implants. A year later, growth of primary MM cells in irradiated SCID mice implanted with human fetal bone was demonstrated. Moreover, it was shown that primary MM cells only grew in the human fetal bone and not in the murine bone marrow or blood [16]. The above-mentioned models are collectively referred to as SCID-hu due to the human tissue that the SCID mouse bears. The tissue need does not have to be fetal bone as fetal liver has also been implanted into SCID mice and also referred to as a SCID-hu model [17]. The SCID-rab model was recently developed to replace the SCID-hu MM models mentioned above, as there were potential ethical and scientific concerns regarding the use of human fetal bone and growth of MM from primary tumor samples and cell lines in an unnatural stem cell-enriched fetal bone. This alternative model was developed in 2004 and consisted of implanting rabbit bones into unconditioned SCID mice, allowing bone engraftment for 6-8 weeks prior to injection with unseparated primary human MM BMMCs or CD138-selected primary MM cells [18]. Graft takes were successful and myeloma cells only grew in the rabbit bone. Different animal model systems have been used to study MM but the choice of the specific model has been based on the goals of the research. While these models are used to study myeloma and its manifestations, they are limited in their usefulness. For example, they are not useful as chemotherapy screening systems in vivo due to the inability to generate large cohorts of animals bearing tumors. The vast majority of primary MM cells from patients do not grow in SCID mice. Most of the time, the tumors grown in vivo are from established MM cell lines. These cell lines come from the most advanced cases of extramedullary myeloma which is not representative of the myeloma seen in most patients that are seen in the clinic. In addition, these tumor cells grow in vitro without the requirement of supportive cells which are critical to the growth of MM in humans.

The models mentioned thus far are heterotransplants. Models in which the cancer is of mouse origin are called murine models. It is appropriate here to mention the mouse plasmacytoma and MM models since prior to the development of human MM, these mouse models were what was available to contribute to our understanding of plasmacytomagenesis and screening of drugs. Experiments in the late 1920s and 1930s tested the relative potency of different carcinogenic agents when applied to the skin and peritoneal cavity of mice, respectively [19, 20]. In 1949, it was shown that tumors were induced in susceptible mouse tissues by the use of various carcinogenic hydrocarbons [21, 22]. One hydrocarbon (or oil) in particular caused two cases of plasma cell leukemias [21]. It was later shown in 1969 by Michael Potter that pristane (a mineral oil) induced mouse plasma cell tumors, secreting monoclonal immunoglobulin (Ig), when housed under conventional conditions [23]. Consequently, the pristane oil-induced mouse plasmacytoma (MPC) model was the most widely used and accepted model to study plasmacytomagenesis. A major benefit of this model that is not found in the current human myeloma models used today is that the BALB/c used to generate the MPCs are immune competent. This model helped scientists better understand cancerous plasma cells; however, there were significant differences when compared to their human counterparts. Most MPCs secreted IgA whereas the majority of human MM secreted IgG [24], and chromosomal analysis of human MM and MPC demonstrated that the Ig heavy chain translocation partners found in human MM were

not shared by the MPC tumors [25]. In 1996, a different murine model was discovered in which plasmacytomas developed spontaneously in aging mice of the C57BL/KaLwRij strain [26]. This model is called the mouse MM (mMM) and a year later, a cell line from the 5T33 model was established [27]. Some, but not all, elderly mMM mice have been found to predominately secrete excessive amounts of monoclonal IgG in the serum and show bone marrow (BM) infiltration with myeloma cells which produce the osteolytic lesions seen in human MM. These murine MM cells have been engineered to express enhanced green fluorescent protein (GFP) allowing for the analysis of homing pattern studies, evaluation of the effects of various therapeutic treatment approaches and imaging of tumor foci in the skeleton in a temporal fashion [28, 29]. However, the GFP-based in vivo model of a hematological malignancy was first shown in a human MM cell line which was transfected with a construct for GFP which allowed for noninvasive, real-time monitoring of the precise localization of tumor lesions and for the assessment of activity of anti-MM therapeutics [30]. The reader is referred to reviews of traditional MM models for additional information [31–33].

Recently, several new MM models have been developed. In order to address the issue of the lack of a human microenvironment in models involving mice as the host for the human MM, Calimeri and colleagues have used a bone-like scaffold as a surface to seed human BM stromal cells (SCs) which were implanted into SCID mice [34]. Three weeks later, autologous MM tumor cells were implanted into the mice and showed successful engraftment within the scaffolds containing the human BMSCs. This model provides the advantage of allowing assessment of effects of drugs on the human MM as well as the nonmalignant supporting cells. Data on two new transgenic MM models were recently published. Activation-induced deaminase (AID)-dependent activation of MYC in germinal center B-cells of VkMYC mice resulted in the progression of benign monoclonal gammopathy to an indolent MM with biological and clinical features characteristic of human MM [35]. These transgenic mice were found to mostly secrete paraprotein of the IgG1 type, plasma cell expansion localized in the murine bone marrow and low hemoglobin concentrations indicating anemia. Additionally, lytic bone lesions, vertebral collapse and decreased bone mineral density were occasionally detected in these VkMYC mice. Plasma cells from this model were also found to be responsive to conventional chemotherapies that are active in MM patients. In contrast to most human MMs which involve the translocation of chromosome 14q32 and specific chromosomal sites including 11q13 [36, 37], the MYC oncogene activation that occurs in this murine model only occurs in less than 5% of human MM [25]. The second transgenic model is of a MGUS/MM phenotype in mice with Eμ-directed expression of the XBP-1 spliced isoform (XBP-1 s) [38]. The transcription factor XBP-1 is a major regulator of the unfolded protein response (UPR) and abundant expression of XBP-1 s has been detected in human MM [39]. MM cells are known to have a well-developed endoplasmic reticulum (ER), as they secrete excessive amounts IgG, and harbor a prominent well-organized Golgi region. The tumor cells are thus heavily reliant on the ER for IgG synthesis and the UPR whose function is to properly construct unfolded proteins and correctly put together misfolded proteins which accumulate under ER stress conditions. The Eμ-*xbp-1 s* transgenic mice

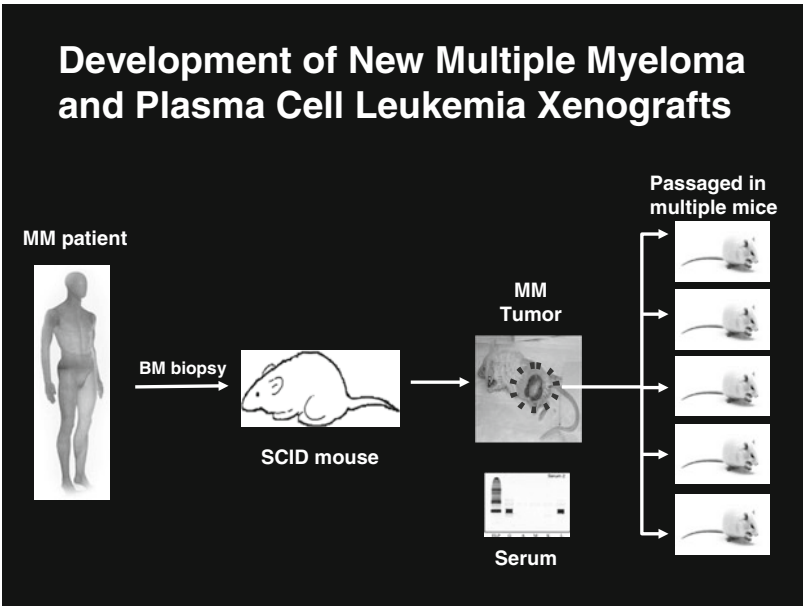


Fig. 16.1 SCID mice are surgically implanted into the left hind limb with a 20–40 mm^3 bone marrow biopsy sample from a MM patient. The use of the biopsy allows implantation of both the malignant population and all of the supporting cells and structures in the BM from the MM patient. This overcomes the disadvantages of using cell lines or fresh BM aspirates which lack these additional elements which are critical to the establishment and growth of human MM

develop elevated serum IgG, subendothelial Ig deposition and lytic bone lesions. This murine model is an additional tool which should help us to further understand the progression of MGUS to MM.

The development of drug resistance remains the most significant problem for myeloma patients. Despite advances in therapy for MM, patients with this disease will ultimately progress from each of their treatment regimens. Thus, it is imperative to optimize the paradigms that will give patients longer lives with reduced side effects as they require many different anti-MM regimens during their course of disease. For this reason, we have attempted to grow and maintain tumors derived from freshly obtained MM bone marrow biopsies and blood from plasma cell leukemia (PCL) patients in SCID mice [40].

The "in vivo" lines we have developed display many features, morphologic and immunologic, typical of human MM and plasma cell leukemia that are especially suited for evaluation of agents in the preclinical setting and are faithful to the sensitivity and resistance of treatments of the patient from which they were derived. Figure 16.1 outlines the procedures used to generate human MM xenografts using this model.

From the growth and passage of the implanted sample, we have established ectopic models of human MM and PCL in SCID mice. The morphology of the tumors growing in SCID mice remains unchanged and is identical to that from the human MM tumor

from which they were originally obtained. A large nuclear to cytoplasmic ratio is a prominent feature of malignant plasma cells. Similarly, the nuclei of the plasma cells growing in SCID mice occupy most of the cells with some cytoplasm located along the internal perimeter of the cell membrane. In these models, we have not only achieved a near 100% success rate at passing these tumors from mouse to mouse, but we are also able to grow MM tumors in a short period of time. Human immunoglobulin G (hIgG) levels are detectable by ELISA after 7 days and a tumor is palpable as soon as 14 days following implantation of the tumor fragment. Notably, the growth pattern of the tumor and the rise in hIgG levels is very similar among different SCID mice bearing the same tumor.

For serial passages of the transplants, tumor-bearing donor mice are sacrificed and tumors aseptically dissected, cut into small tumor fragments and intramuscularly implanted into recipient mice. These tumors have been maintained by serial transplantation for years. The growth rate of each MM tumor varies but the average time to passage into another host is approximately one and a half months. By using an established tumor, we are able to quickly generate large numbers of MM-bearing animals for preclinical studies. Additionally, these solid tumors can be excised, digested into a single cell suspension, and injected subcutaneously or intravenously [40]. If injected intravenously, mice develop ruffled fur and hind limb paralysis, suggesting spinal compression. These models are specifically designed to rapidly and accurately evaluate the anti-MM activity of novel therapies and therapeutic regimens usually within a few weeks following initial administration of the treatment. They have proven highly useful to many pharmaceutical companies, ranging in size from small to large, for the clinical development of different anti-MM treatment strategies.

In contrast to MM cell lines and similar to primary MM patient cells, the MM tumors we have developed do not grow in vitro and thus cannot be maintained indefinitely in cell culture. These tumors can only be maintained via serial in vivo passage in SCID mice. Culture of these tumors in vitro, however, does allow a window of opportunity to test the direct anti-MM effects of anticancer agents as they can survive in culture for a week, providing us the opportunity to evaluate direct short-term anti-MM drug effects. Another difference between these models and traditional MM cell lines is that our tumors predominantly secrete paraproteins of IgG types. Similarly, the majority of patients diagnosed with MM show production of this same type of monoclonal protein. Comparisons of the preclinical to clinical outcomes of drug responses between conventional MM cell lines, which do not secrete IgG, and MM tumor lines, which do secrete IgG, have not been determined in any systematic manner. Whether MM animal models which secrete paraprotein IgG and thus more closely mimic the clinical diagnosis of MM are better predictors of drug responses in humans when compared to MM cell lines grown in animal models is currently unknown.

Data obtained from preclinical studies using our MM animal models have led to many clinical trials. For example, using the LAGλ-1 model, the dosing schedule of liposomal doxorubicin was optimized. Low doses administered once daily on three consecutive days per week were shown to decrease tumor growth and human

paraprotein levels whereas much higher doses given once weekly had no anti-myeloma effects [41]. Moreover, the daily schedule was better tolerated than the weekly regimen. We have also shown that tumor-bearing mice treated with the combination of ATO and bortezomib have markedly decreased tumor volumes and lower paraprotein levels compared to single agent and vehicle-treated mice [42]. A Phase I study evaluated this combination with ascorbic acid. This regimen did not reach dose-limiting toxicity with full doses of both drugs and showed clinical activity [43]. Notably, we have used these models to also evaluate many investigational agents both alone and in combination with other anti-MM agents [44], and these studies have led to many current clinical trials which have shown promising early results [45, 46]. In addition**,** we have recently published data evaluating CEP-18770 [44], a new proteasome inhibitor, as a single agent and in combination with anti-MM agents both in vitro and in vivo (Fig. 16.2), and as a result of these promising results a Phase I trial evaluating CEP-18770 is currently being evaluated.

16.2 Conclusion

It is difficult to predict with high accuracy which preclinical agents demonstrate clinical activity based on the results from the few mouse models of human MM that are available today. It is unrealistic to expect that they will predict the variation in response that occurs in MM patients to chemotherapeutic agents that are currently being tested in various preclinical laboratories and on their way into the clinic. The FDA approval rate for oncology drugs is only approximately 10% and substantially lower than that of pharmaceutical agents in general (21.5% overall) [47]. The low oncology approval rating and lack of drug efficacy in humans suggests that traditional animal cancer models have limited predictive power. Furthermore, it is well established that drug responses to various known anticancer agents differ between patients of the same cancer type. Morphologically similar tumors differ greatly in metabolic, biochemical, and genetic constitution. Human myeloma is known to show marked heterogeneity in terms of its genomic properties [48] and especially its responsiveness to different therapeutic regimens. It is therefore essential to increase the number of MM models available to provide investigators with a variety of genetically distinct tumors, to test novel therapeutic agents alone and in combination with conventional agents, to more closely reflect the clinical heterogeneity of MM. We will then be able to predict with higher accuracy than we currently do the response of patients overall to clinically active anticancer agents and more importantly hopefully individualize use of effective therapies to optimize outcomes for patients with multiple myeloma. Unfortunately, heterotransplantation systems are difficult to establish due to the low tumor take rates and difficulties in obtaining serially transplantable tumors. Our goal is to increase the number of different primary MM samples growing long term in mice to rapidly and accurately test the efficacy of new treatments for patients with MM.

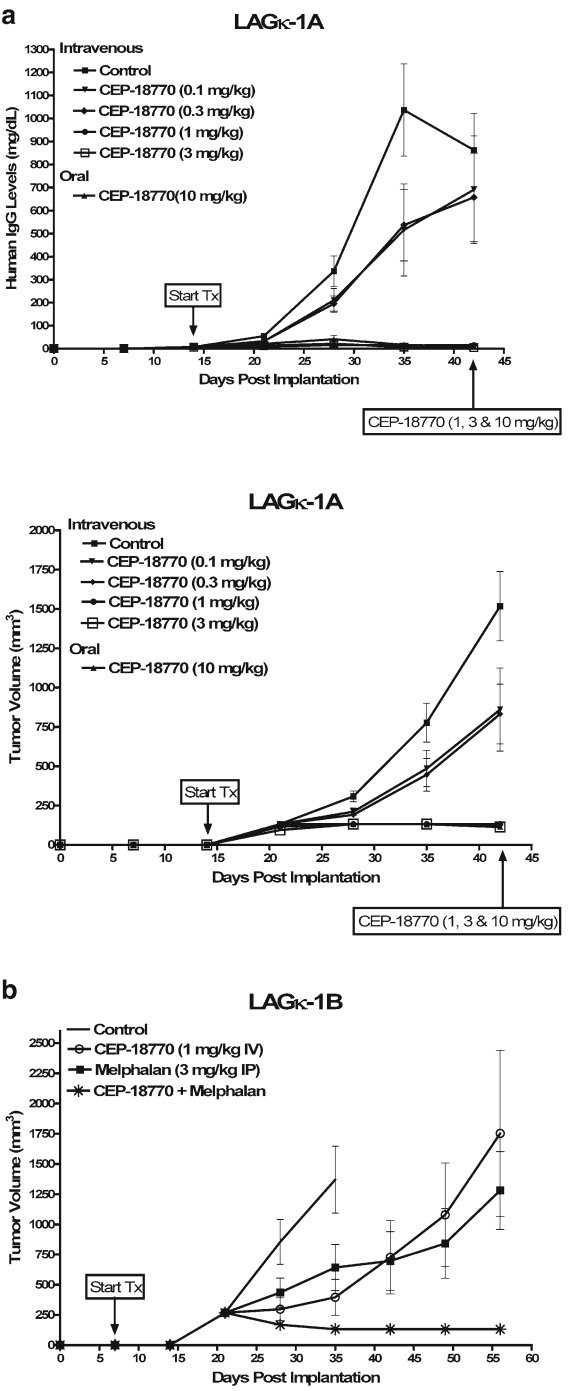
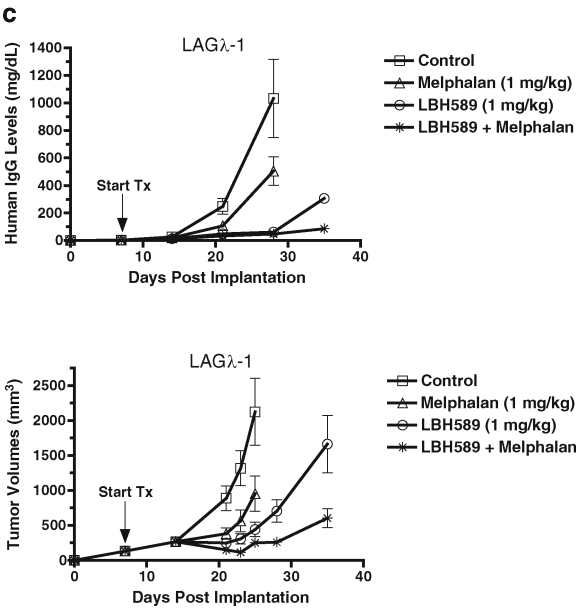


Fig. 16.2 In vivo growth curves of different human MM tumors growing in SCID mice. (**a**) Mice bearing the LAGκ-1A tumor show similar IgG levels and tumor volumes within specific treatment groups and the untreated control group. (**b**) Tumor developed from the same patient as that in Fig. 16.2a, after the patient developed resistance of bortezomib and melphalan, in which mice received a combination of drugs. Mice bearing this tumor (LAGκ-1B) also show similar tumor volume growth curves within a specific treatment group and untreated control group. (**c**) Typical growth curves (IgG and tumor volumes) for the LAGλ-1 tumor

Fig. 16.2 (continued)



Despite the need for additional MM animal models, the lives of many patients have been already improved by the use of the currently available MM models. Studies with alkylating agents, newer anthracyclines, proteasome inhibitors and arsenicals using these models have helped provide the basis for many of the currently used treatment regimens for our patients with MM. Using in vitro MM cell lines, direct or "on target" anticancer effects on MM cells and the corresponding mechanism of action can be efficiently and inexpensively be evaluated. On the other hand, since anticancer drugs will be given to humans, in vitro cancer models do not allow for the evaluation of the indirect or "off target" anticancer and potential toxic effects on organs and organ systems. Thus, in vivo models are needed for this determination. They can be routinely used to provide drug screening systems to identify active anticancer agents. Once active agents are identified, doses, schedules, routes of administration, and the sequencing of drug combinations can be optimized to expedite the evaluation of these compounds in clinical trials. By applying the knowledge gained from the laboratory to the clinic, MM models should provide better treatment options to MM patients on a continuous basis. Additionally, these models are allowing us to explore new targeted therapeutic approaches that will lead to treatments that will target the cancer without damaging healthy cells. Ideally, these targeted approaches will produce more effective therapies with decreased side effects.

References

1. Murphy JB (1912) Transplantation of tissues to the embryo of foreign species. J Exp Med 17:482–493
2. Murphy JB (1914) Factors of resistance to heteroplastic tissue-grafting. J Exp Med 19:513–522
3. Green HSN (1941) Heterologous transplantation of mammalian tumors. J Exp Med 73:461–474
4. Green HSN (1938) Heterotransplantation of human and other mammalian tumors. Science 88:357–358
5. Green HSN (1950) The heterotransplantation of human melanomas. Yale J Biol Med 22:611–620
6. Kutner LJ, Southam CM (1960) Growth of human cancer cells (HEp 2) in newborn rats. Proc Soc Exp Biol Med 104:785
7. Southam CM, Tanzi AF, Ross SL (1966) Growth of primary explants of human cancer in newborn rats. Cancer 19:1670–1682
8. Benjamin I, Pinkerton H (1976) Human breast carcinoma: heterotransplantation to newborn rats. Cancer Lett 1:203–206
9. Miller JFAP (1962) Role of the thymus in transplantation immunity. Ann NY Acad Sci 99:340–354
10. Flanagan SP (1966) Genetic Res 8:295
11. Pantelouris EM (1968) Absence of thymus in a mouse mutant. Nature 217:370–371
12. Raygaard J, Povlsen CO (1969) Heterotransplantation of a human malignant tumor to nude mice. Acta Pathol Microbial Scand 77:758–760
13. Bosma GC, Custer RP, Bosma MJ (1983) A severe combined immunodeficiency mutation in the mouse. Nature 301:527–530
14. Feo-Zuppardi FJ, Taylor CW, Iwato K, Lopez MHA, Grogan TM, Odeleye A, Hersh EM, Salmon SE (1992) Long-term engraftment of fresh human myeloma cells in SCID. Blood 80:2843–2850
15. Urashima M, Chen BP, Chen S, Pinkus GS, Bronson RT, Dedera DA, Hoshi Y, Teoh G, Ogata A, Treon SP, Chauhan D, Anderson KC (1997) The development of a model for the homing of multiple myeloma cells to human bone marrow. Blood 90:754–765
16. Yaccoby S, Barlogie B, Epstein J (1998) Primary myeloma cells growing in SCID-hu mice: a model for studying the biology and treatment of myeloma and its manifestations. Blood 92:2908–2913
17. Namikawa R, Weilbaecher KN, Kanesshima H, Yee EJ, McCune JM (1990) Long-term human hematopoiesis in the SCID-hu mouse. J Exp Med 172:1055–1063
18. Yatta K, Yaccoby S (2004) The SCID-rab model: a novel *in vivo* system for primary human myeloma demonstrating growth of CD138-expressing malignant cells. Leukemia 18:1891–1897
19. Twort CC, Twort JM (1929) The relative potency of carcinogenic tars and oils. J Hyg 29:373–379
20. Twort CC, Twort JM (1937) The fate of mineral oils injected into the peritoneal cavity of mice. J Hyg 38:255–259
21. Rask-Nielsen R (1950) On the susceptibility of the thymus, lung, subcutaneous and mammary tumors in strain street mice to direct application of small doses of four different carcinogenic hydrocarbons. J Hyg 4:108–116
22. Rask-Nielsen R (1950) Types of tumors in the lungs of strain street mice following direct application of large doses of four different carcinogenic hydrocarbons. J Hyg 4:117–123
23. Anderson PN, Potter M (1969) Induction of plasma cell tumours in BALB/c mice with 2,6,1014-tetramethylpentadecane (pristine). Nature 222:994–995
24. Potter M (1997) Experimental plasmacytomagenesis in mice. Hematol Oncol Clin North Am 11:323–347

25. Kuehl WM, Brents LA, Chesi M, Bergsagel PL (1996) Selective expression of one c-myc allele in two human myeloma cell lines. Cancer Res 56:4370–4373
26. Radl J, Van Arkel C, Hopstaken CM, HogenEsch H (1996) Ten-fold increased incidence of spontaneous multiple myeloma in long-term immunosuppressed aging aging C57BL/KaLwRij mice. Clin Immunol Immunopathol 79:155–162
27. Garrett IR, Dallas S, Radl J, Mundy GR (1997) A murine model of human myeloma bone disease. Bone 20:515–520
28. Alici E, Konstantinidis KV, Aints A, Dilber SM, Abedi-Valugerdi M (2004) Visualization of 5T33 myeloma cells in the C57BL/KaLwRij mouse: establishment of a new syngeneic murine model of multiple myeloma. Exp Hematol 32:1064–1072
29. Oyajobi BO, Munoz S, Kakonen R, Williams PJ, Gupta A, Wideman CL, Story B, Grubbs B, Armstrong A, Dougall WC, Garrett RI, Mundy GR (2007) Detection of myeloma in skeleton of mice by whole-body optical fluorescence imaging. Mol Cancer Ther 6:1701–1708
30. Mitsiades CS, Mitsiades NS, Bronson RT, Chauhan D, Munshi N, Treon SP, Maxwell CA, Pilarski L, Hideshima T, Hoffman RM, Anderson KC (2003) Fluorescence imaging of multiple myeloma cells in a clinically relevant SCID/NOD *in vivo* model: biologic and clinical implications. Cancer Res 63:6689–6696
31. Potter M (2003) Neoplastic development in plasma cells. Immunol Rev 194:177–195
32. Vanderkerken K, Asosingh K, Croucher P, Van Camp B (2003) Multiple myeloma biology: lessons from the 5TMM models. Immunol Rev 194:196–206
33. Gado K, Silva S, Paloczi K, Domjan G, Falus A (2001) Mouse plasmacytoma: an experimental model of human multiple myeloma. Haematologica 86:227–236
34. Calimeri T, Battista E, Conforti F, Neri P, Di Martino MT, Rossi M, Foresta U, Piro E, Ferrara F, Amorosi A, Bahlis N, Anderson KC, Munshi N, Tagliaferri P, Causa F, Tassone P (2011) A unique three-dimensional SCID-polymeric scaffold (SCID-synth-hu) model for *in vivo* expansion of human primary multiple myeloma cells. Leukemia 25:707–737
35. Chesi M, Robbiani DF, Sebag M, Chng WJ, Affer M, Tiedemann R, Valdez R, Palmer SE, Hass SS, Stewart AK, Fonseca R, Kremer R, Cattoretti G, Bergsagel PL (2008) AID-dependent activation of a MYC trangsgene induces multiple myeloma in a conditional mouse model of post-germinal center malignancies. Cancer Cell 13(2):167–180
36. Roschke V, Hausner P, Kopantzev E, Pumphrey JG, Riminucci M, Hilbert DM, Rudikoff S (1998) Disseminated growth of murine plasmacytoma: similarities to multiple myeloma. Cancer Res 58:535–541
37. Radl J, Croese JW, Zurcher C, Van den Enden-Vieveen MH, de Leeuw AM (1988) Animal model of human disease. Multiple myeloma. Am J Pathol 132:593–597
38. Carrasco DR, Sukhdeo K, Protopopova M, Sinha R, Enos M, Carrasco DE, Zheng M, Mani M, Henderson J, Pinkus GS, Munshi N, Horner J, Ivanova EV, Protopopov A, Anderson KC (2007) The differentiation and stress response factor XBP-1 drives multiple myeloma pathogenesis. Cancer Cell 11(4):349–360
39. Davies FE, Dring AM, Li C, Rawstron AC, Shammas MA, OConnor SM, Fenton JA, Hideshima T, Chauhan D, Tai IT (2003) Insights into the multistep transformation of MGUS to myeloma using microarray expression analysis. Blood 102:4504–4511
40. Campbell RA, Berenson JR (2008) Animal models of multiple myeloma and their utility in drug discovery. Curr Protoc Pharmacol 40:14.9.1–14.9.22 (unit 14.9. Hoboken, NJ: John Wiley & Sons, Inc.)
41. Campbell RA, Manyak SJ, Yang HH, Sjak-Shie NN, Chen H, Gui D, Popoviciu L, Wang C, Gordon M, Pang S, Bonavida B, Said J, Berenson JR (2006) LAGlambda-1: a clinically relevant drug resistant human multiple myeloma tumor murine model that enables rapid evaluation of treatments for multiple myeloma. Int J Oncol 28(6):1409–1417
42. Campbell RA, Sanchez E, Steinberg JA, Baritaki S, Gordon M, Wang C, Shalitin D, Chen H, Pang S, Bonavida B, Said J, Berenson JR (2007) Antimyeloma effects of arsenic trioxide are enhanced by melphalan, bortezomib and ascorbic acid. Br J Haematol 138(4): 467–478

43. Berenson JR, Matous J, Swift RA, Mapes R, Morrison B, Yeh HS (2007) A phase I/II study of arsenic trioxide/bortezomib/ascorbic acid combination therapy for the treatment of relapsed or refractory multiple myeloma. Clin Cancer Res 13(6):1762–1768
44. Sanchez E, Li M, Steinberg JA, Wang C, Shen J, Bonavida B, Li ZW, Chen H, Berenson JR (2010) The proteasome inhibitor CEP-18770 enhances the anti-myeloma activity of bortezomib and melphalan. Br J Haematol 148(4):569–581
45. Waterman G, Yellin O, Swift R, Mapes R, Berenson JR (2011) A retrospective review of pegylated liposomal doxorubicin (PLD), bortezomib and dexamethasone (DVD) for relapsed and refractory (R/R) multiple myeloma (MM) patients. Ann Hematol 90:193–200
46. Berenson JR, Yellin O, Boccia RV, Nassir Y, Rothstein S, Swift R (2009) A phase I study of oral Melphalan combined with LBH589 for patients with relapsed or refractory multiple myeloma (MM). 2009 ASH Annu Meet Abstr 114(22): Abstract # 1855:736 (December 2009)
47. Kung A (2007) Practices and pitfalls of mouse cancer models in drug discovery. Adv Cancer Res 96:191–212
48. Chapman MA, Lawrence MS, Keats JJ, Cibulskis K, Sognez C, Schinzel AC, Harview CL, Brunet JP, Ahmann GJ, Adli M, Anderson KC, Ardlie KG, Auclair D, Baker A, Bergsagel PL, Bernstein BE, Drier Y, Fonseca R, Gabriel SB, Hofmeister CC, Jagannath S, Jakubowiak AJ, Krishnan A, Levy J, Liefeld T, Lonial S, Mahan S, Mfuko B, Monti S, Perkins LM, Onofrio R, Pugh TJ, Rajkumar SV, Ramos AH, Siegel DS, Sivachenko A, Stewart AK, Trudel S, Vij R, Voet D, Winckler W, Zimmerman T, Carpten J, Trent J, Hahn WC, Garraway LA, Meyerson M, Lander ES, Getz G, Golub TR (2011) Initial genome sequencing and analysis of multiple myeloma. Nature 471:467–472

Index

N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 1: Basic Science*, DOI 10.1007/978-1-4614-4666-8,