3 Molecular Pathogenesis of Multiple Myeloma: Chromosomal Aberrations, Changes in Gene Expression, Cytokine Networks, and the Bone Marrow Microenvironment

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Abstract This chapter focuses on two aspects of myeloma pathogenesis: (1) chromosomal aberrations and resulting changes in gene and protein expression with a special focus on growth and survival factors of malignant (and normal) plasma cells and (2) the remodeling of the bone marrow microenvironment induced by accumulating myeloma cells. We begin this chapter with a discussion of normal plasma cell generation, their survival, and a novel class of inhibitory factors. This is crucial for the understanding of multiple myeloma, as several abilities attributed to malignant plasma cells are already present in their normal counterpart, especially the production of survival factors and interaction with the bone marrow microenvironment (niche). The chapter closes with a new model of pathogenesis of myeloma.

3.1 Survival, Growth, and Inhibitory Factors of Normal Plasma Cells

3.1.1 Survival and Growth Factors of Normal Plasma Cells and Their Generation

Plasma cells are mostly located in the bone marrow where they represent 0.25% of bone marrow mononuclear cells. Generated in the lymph node, due to their rarity, their generation and biology are poorly understood despite extensive studies during the last 10 years (Batista and Harwood 2009; Allen et al. 2007a, b). Naïve B cells entering into lymph node through high endothelial venules are selected by the antigen in the germinal center reaction, yielding selection of B cells with high affinity immunoglobulins and differentiation into memory B cells ($CD20^{+}CD19^{+}CD27^{+}CD38^{-}$) and early plasmablasts ($CD20^{-}CD19^{+}CD27^{++}CD38^{++}$).

The differentiation of B cells into plasma cells involves profound molecular changes yielding a cell able to produce large amounts of immunoglobulins for a long time. Two sets of transcription factors that repress each other are involved in this process (Cobaleda et al. 2007; Calame 2008); see Fig. 3.1). *Activation-dependent induction of Blimp-1*: The guardian of B cell phenotype is PAX5, which induces B cell genes and represses genes as *PRDM1* and *XBP1*, whose gene products – Blimp-1 and XBP1 – are critical for plasma cell generation and survival. BCL6 in association with MTA3 maintains the B cell phenotype and proliferation, down-regulating *PRDM1* expression. In the germinal center, activation of B cells through B cell receptor (BCR), CD40, and/or Toll like receptor (TLR) results in up-regulation of IRF4, down-regulation of BCL6 protein expression, and loss of *PRDM1* repression. This results in down-regulation of *PAX5* and then up-regulation of *XBP1*. In the centrocyte region, stimulation by IL-10, IL-21, or IL-6 results in STAT3 activation yielding *PRDM1* overexpression (Ettinger et al. 2007; Schmidlin et al. 2009).

This results in the full engagement of B cell differentiation into plasmablasts, in particular with the switch from surface to cytoplasmic immunoglobulins, and induction of the unfold protein response driven by XBP1. The detailed hierarchy of this molecular regulation is not fully understood and still a challenging issue.

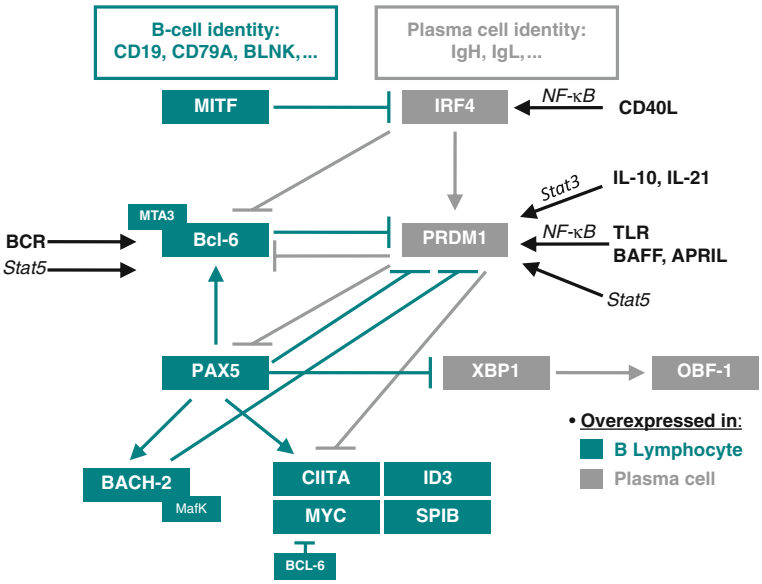


Fig. 3.1 *Plasma cell development*. Transcription factor network regulating B cell and plasma cell identity

Recent data suggest that PAX5 down-regulation and consecutive XBP1 up-regulation are the initial driving events in plasma cell generation independently of Blimp-1 expression (Kallies et al. 2007). Other data indicate a major role of IRF4 whose expression is triggered by NF-kB signaling (Saito et al. 2007).

Plasmablasts exit into peripheral blood and may survive for a short period only unless they are recruited into bone marrow, spleen, or mucosa-associated lymphoid tissues depending on their chemokine receptor expression (Arce et al. 2004; Gonzalez-Garcia et al. 2008; Mei et al. 2009). Expression of sphingosine 1 phosphate receptor 1 (S1P1) is important for the exit of lymph node plasmablasts into blood (Kabashima et al. 2006). In contact with their relevant niche, plasmablasts further differentiate into mature plasma cells that survive independently of antigen for several years yielding a long-term immunity. This explains why treatment with anti-CD20 antibody does not affect the level of circulating immunoglobulin that is insured by these long-term surviving plasma cells (DiLillo et al. 2008). The mechanisms of further differentiation of plasma cells and of homing are partly understood. Homing of plasmablasts into the bone marrow is driven in part by L selectin-induced rolling onto bone marrow endothelial cells, CXCR4 activation by CXCL12 produced by bone marrow stromal cells, as well as by VLA4 expression making adhesion to $VCAM1^+$ bone marrow endothelial cells possible. Recruitment of plasmablasts into mucosa-associated lymphoma tissues is in part mediated by CCR10 expression, making recruitment through CCL28 produced in mucosa tissues possible (Kunkel and Butcher 2003).

These niches provide plasmablasts the factors to survive and further differentiate into long-living mature plasma cells (Tarlinton et al. 2008). CCR10 expressing IgA^+ plasmablasts are mainly recruited to mucosa niche by the CCL28 chemokine (Kunkel and Butcher 2003). In the bone marrow, the plasma cell niche involves SDF-1 producing cells recruiting $CXCR4^+$ plasmablasts and is shared by hematopoietic stem

cells and pre-pro-B cells (Tokoyoda et al. 2004). The rarity of this niche explains the low amount of bone marrow plasma cells and is a matter of regulation of normal immunoglobulin production (Radbruch et al. 2006). "Young" plasma cells have to compete with the "old ones" to establish themselves in a niche (Odendahl et al. 2005). A hallmark of mature plasma cells is their large immunoglobulin secretion, a high expression of the syndecan-1 proteoglycan that is not expressed on B cells, and a lack of most B cell markers except CD19. These plasma cells also largely express CD38.

The intercellular communication signals that are critical to induce this B cell differentiation into plasmablastic cells and plasma cells are poorly known. Plasmablastic cells can be highly expanded in vivo in patients with acute or chronic inflammation. They comprise syndecan-1$^-$ immature plasmablastic cells that can yield syndecan-1$^\pm$ plasmablastic cells (Jego et al. 1999).

We recently developed a three-step in vitro model of generation of polyclonal plasma cells starting from healthy donor's peripheral blood B cells (Jourdan et al. 2009; see Fig. 3.2). It involves a three-step and 10-day culture system comprising a 4-day step 1 to activate and amplify memory B cells using CD40 activation, TLR9 stimulation by CpG oligodeoxynucleotides (ODN) together with IL-2, IL-10, and IL-15. At day 4, the culture medium is removed and cells are cultured for 3 days with IL-2, IL-6, IL-10, and IL-15 to trigger plasmablastic differentiation (step 2), and in a final 3-day step 3 with IFN-α, IL-6, and IL-15 to trigger plasma cell differentiation. This model allows a better understanding of the mechanisms controlling survival of plasmablastic cells in the bone

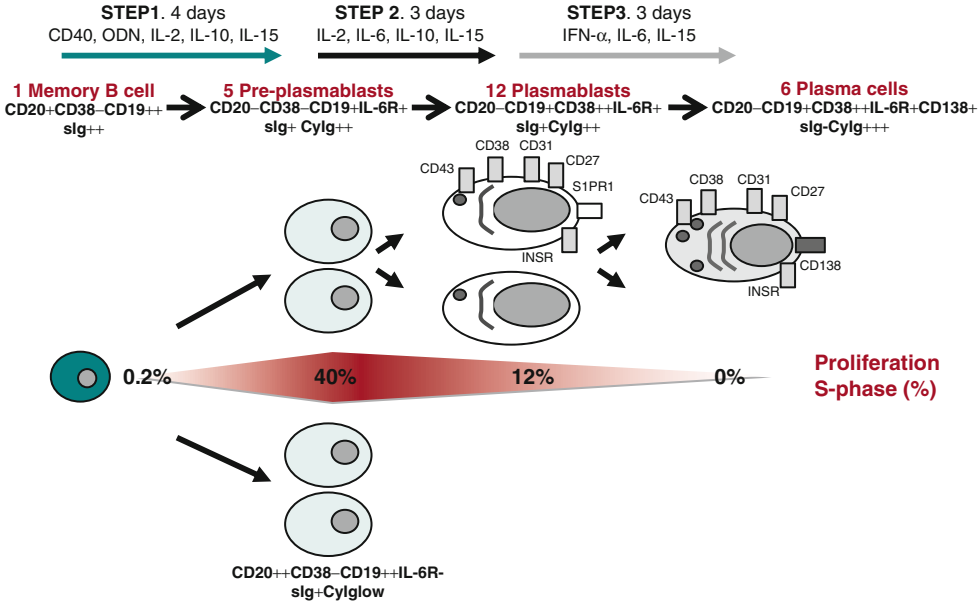


Fig. 3.2 *In vitro generation of plasma cells*. A three-step culture system allows the generation of plasma cells from peripheral blood memory B cells

marrow. A first requirement to induce plasma cell differentiation is the abrogation of CD40 stimulation. A second requirement is the activation of STAT3 through different cytokines as IL-10 and IL-6, yielding induction of *PRDM1*. A major role of IL-6 for the survival of plasmablasts from patients with reactive plasmacytosis was demonstrated by Jego et al. (1999). This property of IL-6 is not surprising since the IL-6 gene was initially cloned in 1988 as a B cell differentiation factor (Yamasaki et al. 1988). In addition, transgenic mice expressing an IL-6 gene driven by an Eμ promoter develop massive polyclonal plasmacytosis (Suematsu et al. 1989), whereas IL-6 knockout mice have a defect in the production of high affinity antibodies (Kopf et al. 1994). IL-21 is also a major cytokine driving plasma cell generation (Ozaki et al. 2002). For an overview of signal transduction pathways in normal and malignant plasma cells, see Fig. 3.3.

3.1.2 Inhibitory Factors Expressed by Normal Plasma Cells

Given the frequently long time from first diagnosis of early-stage plasma cell dyscrasias to overt myeloma and the mostly low proliferation rate of multiple myeloma cells (see below; Witzig et al. 1999), we hypothesize these to express a novel class of inhibitory factors of potential prognostic relevance. Due to their expression and ability to inhibit proliferation of myeloma and memory B cells (Ro et al. 2004; Kersten et al. 2005), bone morphogenic proteins (BMPs)

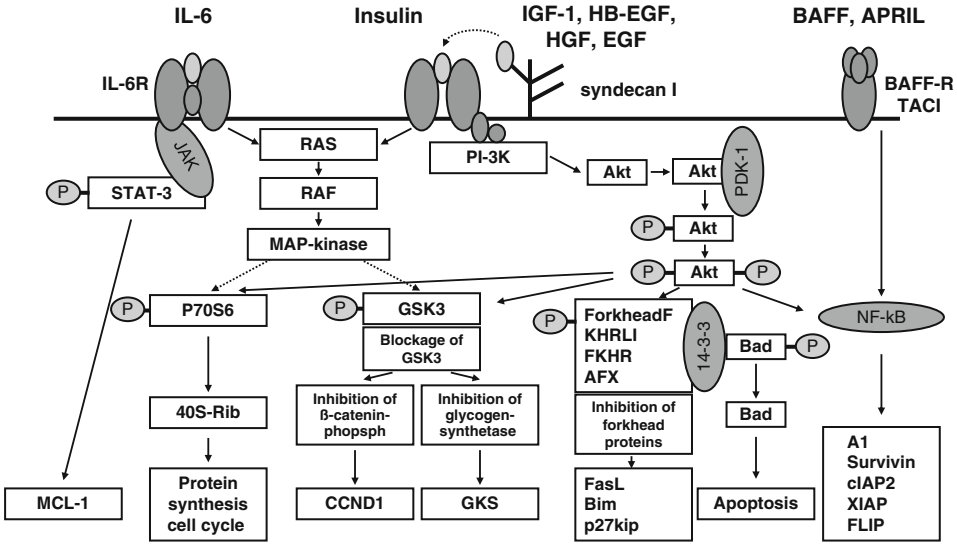


Fig. 3.3 *Signal transduction pathways in normal and malignant plasma cells*. The main signal transduction pathways in plasma cells comprise JAK/STAT signaling, MAPK-signaling, PI3K-signaling, and signaling via NF-kB. Syndecan-1 is a hallmark of normal and malignant plasma cells. It acts in concentration heparin-binding growth and survival factors (including IGF-1, HGF, BAFF, APRIL) at the cell surface and thus facilitates the interaction with the respective receptors. Insulin is a growth factor for normal plasma cells that acts via Insulin-R, and additionally an insulin/IGF1R hybrid receptor in malignant plasma cells. Inhibitory factors like BMP6 physiologically expressed by plasma cells act in terms of checks and balances on this network (not shown) (Modified from Klein et al. 2003)

represent possible candidates. Of these, BMP6 is the only BMP expressed by normal and malignant plasma cells (Seckinger et al. 2009; Zhan et al. 2002). Its expression is significantly lower in proliferating myeloma cells, myeloma cell lines, or plasmablasts. BMP6 significantly inhibits proliferation of myeloma cell lines, survival of primary myeloma cells, and in vitro angiogenesis. High BMP6-expression in primary myeloma cell samples delineates significantly superior overall survival for patients undergoing high-dose chemotherapy independent of conventional prognostic factors (ISS-stage, beta-2-microglobulin; Seckinger et al. 2009). It likewise stimulates osteoblast differentiation (Ebisawa et al. 1999), osteoclast development (Wutzl et al. 2006), and bone formation (Cheng et al. 2003).

BMPs are members of the transforming growth factor-β superfamily, and act through binding to two different types of serine/threonine kinase receptors. Three type I receptors bind BMPs: activin-like kinase-2, (Alk-2, ACVR1), -3 (Alk-3, BMPR1A), and -6 (Alk-6, BMPR1B). Likewise, three type II receptors have been identified, i.e., BMP receptor II (BMPR2), activin type II receptor (ActRII, ACVR2), and activin type IIB receptor (ActRIIB, ACVR2B; Ebisawa et al. 1999). Both, type I and type II receptors are required for signaling (Kawabata et al. 1998). All BMPs use BMPR2, but utilize different BMP type I receptors. BMP6 preferably binds to ACVR1 (Ro et al. 2004). Intracellular BMP-signals are transduced mainly by small mothers against decapentaplegic proteins (SMADs). Alternate BMP-signaling pathways include prostanoid-generation via COX-2 (Ren et al. 2007) and MAPK-dependent activation of p38 or the Ras- and Erk-pathway (Nohe et al. 2004; Du et al. 2007). Both pathways have been reported to be present in myeloma cells (Trojan et al. 2006; Hoang et al. 2006).

BMP, and especially BMP6, are thus of high interest as a novel class of inhibitory and bone formation stimulating factors expressed already by normal plasma cells.

3.2 Chromosomal Aberrations

3.2.1 Background and Methods

A plethora of numerical and structural aberrations can be detected in myeloma cell samples of almost all patients, especially if CD138-purified plasma cells are used (Magrangeas et al. 2005; Kuehl and Bergsagel 2002; Chiecchio et al. 2006; Barlogie et al. 1985; Latreille et al. 1980; Tienhaara and Pelliniemi 1992; Drach et al. 1995; Flactif et al. 1995; Fig. 3.4; Table 3.1). Chromosomal aberrations lead to changes in gene and protein expression causing malignant properties of myeloma cells (Magrangeas et al. 2005), exemplified by aberrant expression of growth and survival factors (Sect. 3.6) but can likewise appear as epiphenomenon.

Three *methods* routinely used to assess chromosomal aberrations in multiple myeloma: (1) *metaphase cytogenetics* (mCG) allow the simultaneous assessment of aberrations of the whole set of chromosomes, but is largely unable to detect small changes or such in terminal regions (e.g., translocation t(4;14); Hallek et al. 1998). Importantly, for detection of aberrations, this method prerequisites myeloma cells to proliferate to obtain metaphases and therefore measures the frequency of aberrations in *proliferating* myeloma cells. mCG showed an increase in the number of aberrations detected in early- vs. late-stage patients and relapsed disease (Hallek et al. 1998). However, this basically reflects the increased proliferation rate in later stages (Hose et al. 2010). Using proliferation-independent methods, i.e., (2) interphase fluorescence in situ hybridization (iFISH; Drach et al. 1995 et seqq.) iFISH (Drach et al. 1995; Flactif et al. 1995; Nishida et al. 1997; Fonseca et al. 2001b; Avet-Loiseau et al. 1998) and (3)array-based comparative genomic hybridization (aCGH), an increasing frequency of aberrations from early-stage plasma cell dyscrasia to overt and

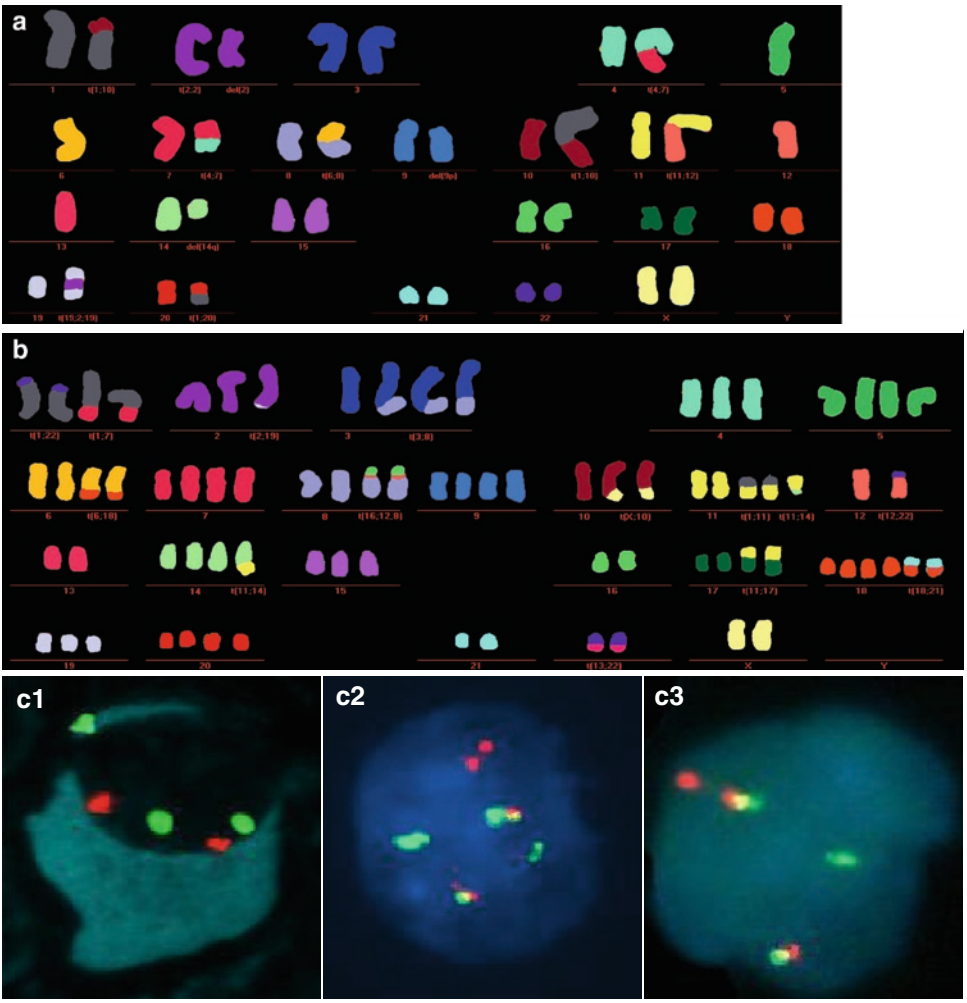


Fig. 3.4 *Metaphase multicolor-FISH*. (**a**) Non-hyperdiploid karyotype with several structural (translocations t(1;10), t(2;2), t(4;7), t(6;8), t(11;12), t(19;2;19), t(1;20)) and numerical (deletion of chromosomes or chromosomal regions 5, 13, and 14q, respectively). (**b**) Hyperdiploid karyotype with characteristic gain of odd numbered chromosomes, including 5, 9, 15, as well as several structural aberrations, including the recurrent translocation t(11;14), as well as nonrecurrent translocations, e.g., t(11;17) and t(1;11). (**c**) Frequent chromosomal aberrations as detected by iFISH. (**C1**) Gain of 11q13 (*green*), normal copy number of 9q34 (*red*). (**C2**) Translocation t(11:14). 11q13 (*green*), 14q32 (*red*). (**C3**) Translocation t(4;14). 4p16 (*green*), 14q32 (*red*)

relapsing myeloma has not been shown. iFISH in CD138-purified plasma cells is currently the workhorse of assessment of (prognostic) chromosomal aberrations and of clonal heterogeneity in terms of presence of subclones (Fig. 3.4, and see below). Before *iFISH* can be used, it is necessary to identify recurrent chromosomal aberrations to generate specific probes. *aCGH* does not have this prerequisite and allows assessment of copy number changes for hundreds of thousands of loci (Carrasco et al. 2006), but does not allow the detection of (prognostically

Table 3.1 Frequency of chromosomal aberrations in multiple myeloma (%)

	iFISH			mCG
	Neben et al. (2010) $n=312$[a]	Avet-Loiseau et al. (2007) $n=1{,}000$[a]	Chiecchio et al. (2006) $n=792$[a]	Chiecchio et al. (2006) $n=213$
Hyperdiploidy	57	40	56	62
Non-hyperdiploidy	43	60	44	39
IgH-translocation (any)	n.a.	n.a.	45	52
t(4;14)	13	14	12	n.a.
t(11;14)	19	21	15	15
t(6;14)	n.a.	n.a.	n.a.	2
t(14;16)	2	n.a.	n.a.	3
t(14;20)	n.a.	n.a.	n.a.	4
Myc-translocations	n.a.	13	n.a.	n.a.
Deletion 17p13	10	11	9	n.a.
Deletion 13q14	46	45	48	48
1q21+	36	40	n.a.	n.a.

n.a. not assessed

[a]Different numbers of assessed patients; maximal number given (Neben et al. 2010; Avet-Loiseau et al. 2007; Chiecchio et al. 2006)

relevant) balanced translocations (e.g., translocation t(4;14)).

3.2.2 Types of Chromosomal Aberrations

Chromosomal aberrations in multiple myeloma can be grouped in (1) *structural* aberrations (mostly translocations, especially IgH-translocations), and (2) *numerical* aberrations of single chromosomes or chromosomal regions (e.g., deletion 13q14), or changes in *ploidy*, i.e., deviations from the diploid karyotype (aneuploidy). The latter are grouped according to the number of chromosomes: "hypodiploidy" (≤45; karyotypes with loss of Y-chromosome as single aberration are not considered abnormal), "pseudodiploidy" (46–47), "near tetraploidy" (≥75), and "hyperdiploidy" (HRD, 48–74; Chiecchio et al. 2006). Hypodiploid, near-tetraploid, and pseudodiploide karyotypes are summarized as non-hyperdiploid (non-HRD), in contrast to HRD. Both represent a broad category each comprising about 50% of abnormal karyotypes (Magrangeas et al. 2005): Hyperdiploid karyotypes show rather "global" changes in terms of *numerical* aberrations (gains), especially of the odd chromosomes 3, 5, 7, 9, 11, 15, 19, and 21. To the contrary, non-HRD karyotypes are mostly characterized by structural aberrations (Magrangeas et al. 2005). Frequently, these are IgH-translocations. In analogy to conventional karyotyping, iFISH can be applied to classify in HRD/non-HRD using a combination of frequently altered chromosomal regions as surrogate (Chiecchio et al. 2006; Wuilleme et al. 2005; Cremer et al. 2005). An example is to classify as HRD if at least two regions on chromosome 5, 9, and 15 are gained (Wuilleme et al. 2005). Alternatively, a value of (+1), (−1), and 0 is attributed for gain, loss, and lack of change for each if the regions 6q21, 8q21, 9q34, 11q23, 13q14, 15q22, 17p13, 19q13, and 22q11 are subsequently summed. For the resulting "copy-number score" (CS; Hose et al. 2004, 2005; Cremer et al. 2005), a value of CS ≥1 is defined as HRD, all others as non-HRD. The ploidy stage (HRD or non-HRD) usually does not change during disease progression (Chng et al. 2006).

Two further ways to classify chromosomal aberrations from a theoretical point of view are (1) whether they exclude each other ("*disjunct aberration*") or not ("*non-disjunct aberrations*"), and (2) whether they are involved in the initial pathogenesis ("*etiopathogenetic aberrations*"), the latter in most cases disjunct (e.g., t(11;14) and t(4;14)), or *additive aberrations* (non-disjunct, e.g., deletion of 17p).

3.2.3 Association of Chromosomal Aberrations

The appearance of several chromosomal aberrations is correlated: A t(4;14) or t(14;16) is in 85–90% of patients associated with a deletion of chromosome 13q14 (Kuppers and Dalla-Favera 2001; Keats et al. 2003; Fonseca et al. 2001a). A deletion 13 can be found in 85% of non-HRD malignant plasma cells, but in 30–35% of HRD malignant plasma cells (Smadja et al. 2001; Santra et al. 2003). Myeloma cells carrying a t(11;14), t(14;16), or t(4;14) are mostly non-HRD (Fonseca et al. 2003b; Magrangeas et al. 2005), those with nonrecurrent 14q32 translocations more frequently HRD. Avet-Loiseau et al. found an association between del(13) and t(4;14), del(17p) and del(13), but not between del(17p) and t(4;14) (Avet-Loiseau et al. 2007). Respective associations are not described for gains of 1q21 or losses of 17p13, see below.

3.2.4 Clonal, Subclonal, and Progression-Related Aberrations and Chromosomal Instability

Chromosomal aberrations can appear in different percentages within the malignant plasma cell population of a given patient. Whereas IgH-translocations as t(4;14) or ploidy state usually appear in the majority of myeloma cells, the frequency of malignant plasma cells in which a deletion 13q14 can be detected varies between 20% and 100% (Magrangeas et al. 2005); the same holds true for deletion of 17p13 or gains of 1q21 (Cremer et al. 2005). If one chromosomal aberration appears in ≥70% of myeloma cells whereas another only in a smaller percentage of this population (20–60%), a so-called subclonal aberration is present. Their appearance can be seen as a sign for an evolution of the malignant plasma cell clone (Cremer et al. 2005), in which the subclonal aberration appeared after the clonal aberration (Fig. 3.5). Neither the absolute number of chromosomal aberrations nor presence of subclonal aberrations tested by iFISH were significantly different between myeloma cells

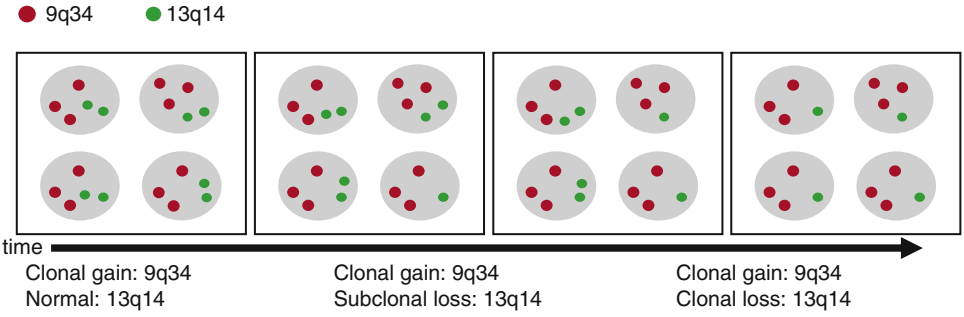


Fig. 3.5 *Subclonal aberrations and chromosomal instability*. Initially (*left*) a clonal gain of 9q34 (*red*) and a normal copy number regarding 13q14 (*green*) are present in all four depicted nuclei (*grey*). With time, a loss of 13q14 appears in a subfraction of myeloma cells (*middle-left*, 25%). This fraction increases (*middle-right*, 50%) until it has become a clonal aberration (100%, *right*). The detection of a subclonal aberration can be seen as an indicator for a present or past clonal instability

showing a gene expression–based proliferation index above vs. below the median (Hose et al. 2011). The appearance of some chromosomal aberrations seems to be associated with an evolution of the malignant plasma cell clone: Gains of 1q21, e.g., are found in none of 14 individuals with MGUS, 43% (206/479) of newly diagnosed and 71% (32/45) of relapsing myeloma patients, as well as in 91% (21/23) of investigated human myeloma cell lines (Hanamura et al. 2006). The percentage of myeloma cells carrying a $1q21^+$ as well as the number of copies of 1q21 within myeloma cells of a given patient increase with disease progression. 1q21-aberrations are frequent in terminal malignant diseases, e.g., in non-Hodgkin lymphoma (Le et al. 2001; Itoyama et al. 2002), Wilms-tumor (Lu et al. 2002), Ewing-sarcoma (Hattinger et al. 2002), ovarian cancer (Cheng et al. 2004), and breast cancer (Cheng et al. 2004; Zudaire et al. 2002). Malignant plasma cells of patients harboring a disease progression–associated gain of 1q21 or deletion of 13q14.3 show a significantly higher gene expression-based proliferation index, whereas patients with gain of chromosome 9, 15, or 19 (hyperdiploid samples) show a significantly lower one, see below (Hose et al. 2011).

It seemed logical that the multitude of chromosomal aberrations, the increase of their percentage in mCG from MGUS to relapsing myeloma, and the presence of subclonal aberrations could be taken as evidence of an ongoing chromosomal instability. However, as detailed above, only in the proliferation-dependent mCG an increase of the frequency of aberrations can be found. This has not been documented for proliferation-independent methods like iFISH. It thus seems that at least on a macroscopic scale, there might have been a chromosomal instability during a period of myeloma development, but there is currently no hard evidence that this process is continuously ongoing. This picture might, however, change, when high-resolution techniques like deep sequencing become available.

3.2.5 Prognostic Relevance of Chromosomal Aberrations

Several chromosomal aberrations show prognostic relevance (see Table 3.2). Already presence of an abnormal karyotype in mCG and detection of abnormal metaphases are associated with shorter survival in multiple myeloma (Chiecchio et al. 2006).

iFISH allows a risk stratification with presence of a translocation t(4;14) and/or deletion of 17p13 being the best-documented adverse prognostic factors (Avet-Loiseau et al. 2007; Chiecchio et al. 2006; Keats et al. 2003; Fonseca et al. 2003a; Moreau et al. 2002; Chang et al. 2004). Of etiology-associated aberrations (e.g., IgH-translocations), the translocation t(4;14) present in about 15% of patients represents a specific disease entity and is an independent risk factor despite conventional or high-dose treatment (Avet-Loiseau et al. 2007; Chiecchio et al. 2006; Keats et al. 2003; Fonseca et al. 2003a; Moreau et al. 2002; Chang et al. 2004). Treatment with bortezomib or lenalidomide containing regimen seems to reduce the negative prognostic impact of this aberration (Barlogie et al. 2008; San Miguel et al. 2008; Avet-Loiseau et al. 2009; Knop et al. 2009; Reece et al. 2009).

Regarding aberrations associated with disease progression, deletion of 17p13 (Avet-Loiseau et al. 2007; Chiecchio et al. 2006), gains of 1q21 (Avet-Loiseau et al. 2007; Hanamura et al. 2006), and deletions of 13q14 in univariate analyses are associated with adverse prognosis (Avet-Loiseau et al. 2007; Chiecchio et al. 2006; Neben et al. 2010). Different results are published regarding multivariate analyses (Neben et al. 2010; Avet-Loiseau et al. 2007). If adjusted for presence of deletion 17p and t(4;14), deletion of 13q14.3 is no longer considered to define adverse risk (Neben et al. 2010; Avet-Loiseau et al. 2007). Deletion of 17p13 remains an adverse prognostic factor in multivariate analyses. It likewise remains an adverse

Table 3.2 Prognostic relevance of chromosomal aberrations as detected by mCG and iFISH: Patients treated with high-dose therapy and autologous stem cell transplantation (Neben et al. 2010; Avet-Loiseau et al. 2007) and patients with conventional as well as high-dose therapy and autologous stem cell transplantation (Chiecchio et al. 2006), respectively.

Aberration	Neben et al. (2010)		Avet-Loiseau et al. (2007)		Chiecchio et al. (2006)					
	iFISH 36 months survival		iFISH 41 months survival		mCG Median survival		iFISH & mCG[b] Median survival		iFISH (all) Median survival	
	(%)	P	Months	P	Months	P	Months	P	Months	P
Abnormal karyotype	n.a.	n.a.	n.a.	n.a	24 vs. 45	0.001	–	–	–	–
Abnormal metaphases	n.a.	n.a.	n.a.	n.a	12 vs. 45	<0.001	–	–	–	–
Del 13q	72 vs. 82	0.037	68 vs. 83	<0.001	15 vs. 50	<0.001	24 vs. n.r.	<0.001	29 vs. 47	<0.001
Any IgH-TL	n.a.	n.a.	n.a.	n.a	24 vs. 41	0.038	–	–	–	–
t(4;14)	49 vs. 82	0.005	41.3[a] vs. 79	<0.001	9 vs. 41	<0.001	19 vs. n.r.	0.004	19 vs. 44	0.002
t(11;14)	79 vs. 77	0.855	80 vs. 74	0.28	n.e. vs. 33	0.787	n.r. vs. 33	0.540	n.r. vs. 36	0.229
t(14;16)	n.a.	n.a.	n.a.	n.a.	16 vs. 40	0.354	–	–	–	–
t(14;20)	n.a.	n.a.	n.a.	n.a.	7 vs. 40	0.109	–	–	–	–
Deletion 17p	50 vs. 81	<0.001	22[a] vs. 75	<0.001	15 vs. 41	0.048	21 vs. 40	0.069	19 vs. 43	<0.001
Hypodiploid	n.a.	n.a.	n.a.	n.a.	21 vs. 40	0.064	–	–	–	–
Non-hyperdiploid	51 vs. 77	0.041	70 vs. 82	0.006	21 vs. 41	0.003	26 vs. n.r.	0.036	33 vs. 47	0.041

Prognostic role of presence of the respective aberration vs. all patients without presence of the respective aberration

TL translocation

[a]Months survival (instead of %)

[b]Simultaneous

prognostic factor for bortezomib- and lenalidomide-based protocols (Knop et al. 2009; Reece et al. 2009).

Many investigations have shown the prognostic relevance of chromosomal aberrations to be independent of clinical parameters, in particular beta-2-microglobulin. Combining these parameters results in powerful prognostic models, in particular those of Facon et al. (beta-2-microglobulin and deletion 13; Facon et al. 2001), Avet-Loiseau et al. (model including t(4;14), del (17p), and serum beta-2-microglobulin >4 mg/dL Avet-Loiseau et al. 2007), or Neben et al. (model including t(4;14), del (17p), and ISS-stage; Neben et al. 2010).

3.3 Changes in Gene Expression in Multiple Myeloma

Multiple myeloma cells harbor a high median number of chromosomal aberrations (Cremer et al. 2005; Fonseca et al. 2004) as discussed above, and multiple changes in gene expression compared to normal bone marrow plasma cells (Andersen et al. 2009, 2010; Zhan et al. 2002, 2006). This molecular heterogeneity is thought to transmit into the very different survival times ranging from a few months to 15 or more years (Barlogie et al. 2006), with a median survival after conventional treatments of 3–4 and 5–9 years after high-dose melphalan treatment followed by autologous stem cell transplantation (Harousseau and Moreau 2009; Barlogie et al. 2008). On a molecular level, it seems that many and multiple myelomas exist (Fonseca 2003).

Gene expression profiling performed on $CD138^+$ purified myeloma cells allows assessing expression of (almost) all genes simultaneously without the need of a preselection of interesting genes or regions. Profiling of gene expression can be used (1) to classify patients due to molecular entities (mostly based on unsupervised clustering algorithms grouping patients according to the similarity of their expression profile), (2) to assess progression of pathophysiologically relevant target genes (e.g., aurora-kinase), (3) in expression and (to a certain extent) molecular entity–based risk assessment.

3.3.1 Gene Expression–Based Classifications in Myeloma

Three gene expression–based classifications delineate molecular groups in myeloma: the "molecular classification" based on differential gene expression in which three of seven groups ("proliferation," MAF-expression, and MMSET-overexpression) show different survival (Zhan et al. 2006), the TC-classification based on translocations and D-type cyclin (CCND) without prognostic relevance (Bergsagel and Kuehl 2005; Bergsagel et al. 2005), and the EC-classification based on chromosomal aberrations and resulting changes in gene expression with only one of four groups (t(4;14) and FGFR3-expression) showing adverse prognosis (Hose et al. 2004, 2005). Biological classifications likely remain relatively stable in contrast to prognostic factors prone to change with different treatment schedules (see below).

The molecular classification of Shaughnessy et al. (Zhan et al. 2006; groups denoted MS, MF, PR, Hy, D1, D2, LB) is based on unsupervised clustering and prediction of clustered groups, whereas the TC-classification by Bergsagel et al. (Bergsagel et al. 2005; groups denoted TC1-7) is centered on the hypothesis that CCND-expression is an early unifying event in multiple myeloma. The EC-classification delineates groups based on expression of CCND and underlying chromosomal aberrations. In EC1.1 and EC1.2, aberrant expression of CCND1, mediated by a gain of 11q13 (the CCND1-locus; Hoechtlen-Vollmar et al. 2000) in EC1.1, or a translocation involving this locus in EC1.2 (Specht et al. 2004;

Wlodarska et al. 2004) is present. Patients in EC1.1 and EC2.1 are almost all hyperdiploid, patients in EC1.2 (mostly) and EC2.2 (all) non-hyperdiploid. In groups EC2.1 and EC2.2, myeloma cells overexpress of the "physio logic" CCND2 involved in the proliferation of plasma cell precursors (i.e., polyclonal plasma cells), and expressed at a low level in normal bone marrow plasma cells. EC2.1 comprises patients with a hyperdiploid karyotype and few patients with rare translocations indicated by the respective expression pattern (e.g., t(14;16), MAF, (4/128), t(14;20), MAFB, (1/128), and FGFR2 (1/128)), and patients with t(4;14) without FGFR3 overexpression (3/128). EC2.2 is characterized by the presence of the translocation t(4;14) and FGFR3 overexpression. CCND2-overexpression seems to be correlated with hyperdiploidy, or triggered by aberrations in physiological plasma cell proliferation pathways like MAF (Hurt et al. 2004) or APRIL/TACI (via MAF; Moreaux et al. 2005). CCND3 expression does not show significant differences between normal bone marrow plasma cells, polyclonal plasma cells, or any of the groups. As an aberrant expression of CCND does not seem sufficient for oncogenic transformation, it is intriguing that in EC2.1 myeloma cells carry a higher number of aberrantly expressed growth factors compared to low (EC1.1) or high (EC1.2) intrinsic CCND1 expression. Therefore, intrinsic expression of CCND might mimic the effect of growth factor stimulation, thereby reducing the dependence of myeloma cells on external stimuli for proliferation and survival.

Despite methodological differences, in all classifications (1) a group with translocation t(4;14) (MS, TC7, EC2.2) and *MMSET* (with or without *FGFR3* expression) is identified and (2) a group with translocation t(11;14)/t(11;v) with high *CCND1* overexpression (EC1.2, TC2, subdivided in D1, D2 (*CCND1* or *CCND3* overexpression)). EC1.1 corresponds with TC3 (low *CCND1*, hyperdiploid), but correlates low *CCND1* overexpression with gain of 11q13 detected by iFISH. EC1.1 together with EC2.1 corresponds with Hy (hyperdiploid). EC2.1 also comprises patients with rare translocations like the MAF-translocations (the latter form separate groups, i.e., MF, TC8) or t(4;14) without *FGFR3* overexpression. We also observed simultaneous *CCND1* and *CCND2* expression as defining TC4, but interpret this either as an evolving aberration $11q13^{+}$ (on the background of physiological *CCND2* expression, which is down-regulated simultaneously with *CCND1* up-regulation), or the presence of two (sub) clones. No correspondence with our groups could be found for TC6 (no *CCND*), as all patients expressed at least one of the CCND, LB (low bone disease), as it was not significantly different distributed between the groups, and PR (proliferation), which seems to be a characteristic acquirable in all groups.

Taken together, gene expression profiling can be used to delineate different groups in myeloma. Some of these represent different entities, but it remains to be shown which are exclusive (disjunct), and which features can appear independent of delineated groups, e.g., emerging of a proliferative geno- and phenotype.

3.3.2 Gene Expression and Risk Stratification

Risk stratification by gene expression profiling is applied using four different strategies: (1) grouping multiple myeloma into "molecular groups" (entities, Sect. 3.4.1) subsequently investigating differences in survival between these groups, (2) assessing expression of a gene representing a potential therapeutic target and investigate its prognostic relevance, (3) assessing surrogates of biological variables and their respective prognostic relevance, and (4) assessing (high) risk based on association of gene expression with survival. The second possibility is exemplified by expression of Aurora-A

(Hose et al. 2009b) delineating significantly inferior survival in two independent cohorts of patients undergoing high-dose chemotherapy, independent from conventional prognostic factors. Gene expression profiling could here allow selecting (only) patients with aurora-kinase expression, which in turn have an adverse prognosis, for treatment with aurora-kinase inhibitors. The third possibility is exemplified by a gene expression–based proliferation index (see Sect. 3.5). Proliferation of malignant plasma cells, as determined by several methods, has been shown to be a strong adverse prognostic factor (Boccadoro et al. 1984; Greipp et al. 1988, 1993; San Miguel et al. 1995; Gastinne et al. 2007), independent of clinical prognostic factors, e.g., beta-2-microglobulin (Greipp et al. 1993), and can likewise be assessed by gene expression–based proliferation indices (Zhan et al. 2002, 2006; Bergsagel and Kuehl 2005; Bergsagel et al. 2005; Hose et al. 2011); see below). The fourth strategy comprises the high risk-scores of the University of Arkansas for Medical Sciences (UAMS; 17/70 genes; Shaughnessy et al. 2007) and the Intergroup Francophone du Myélome (IFM; 15 genes; Decaux et al. 2008) by building a score over a set of genes associated with survival. Both scores allow delineating a small group of patients (13% and 25%, respectively) with very adverse prognosis in the IFM and total therapy 2 (TT2-) dataset (both not including bortezomib), whereas in the TT3-cohort only the UAMS-score remains significant in univariate analysis. Thus, the UAMS-score remains its prognostic relevance if bortezomib is added to the treatment regimen (TT2 vs. TT3; Shaughnessy et al. 2007; Decaux et al. 2008). In relapsed patients treated with bortezomib within the APEX, SUMMIT, and CREST trials ($n = 188$), both scores significantly delineate different outcome, whereas in patients treated with dexamethasone within these trials ($n = 76$), only the UAMS-score significantly delineates a high risk group. No data are currently published in terms of independence of these scores of lenalidomide treatment.

3.4 Proliferation and Cell Cycle Regulation

3.4.1 "Potential to Proliferate" of Normal Plasma Cells

Cell cycle progression is regulated by several classes of cyclin-dependent kinases and their inhibitors (Sherr and Roberts 1999). Following Murry (2004), three basic levels of cell cycle regulation can be delineated: (1) The "cell cycle machinery" mediating the continuing fluctuations of cyclin-levels and activity of associated Cdk, (2) the subsequent targets of this machinery (DNA-replication, mitosis), and (3) signal transduction pathways regulating this machinery in response to external stimuli (Murray 2004). Signal transduction pathways of several growth and survival factors converge on CCND, crucial for G_0/G_1-S progression.

Bone marrow plasma cells have the "*potential to proliferate*." In contrast to their precursors (see Sect. 3.2.1), normal bone marrow plasma cells do not proliferate (Witzig et al. 1999; Drewinko et al. 1981; Hose et al. 2011) but have the "potential to proliferate": They express necessary parts of the cell cycle machinery, e.g., CDK4/6, but likewise cell cycle breaks, e.g., Kip/Cip (p21, p27) and INK4-family members (p18). Molecular integration of pro-proliferative (e.g., CCND2-expression due to growth factor stimulation, e.g., via TACI/c-maf) and thus CCND2/CDK4/6 promotion of G_0/G_1-transgression and anti-proliferative signals including a cell cycle arrest as part of the terminal B cell differentiation (Klein et al. 2003), i.e., *BCL6*-expression necessary for proliferation being suppressed by *PAX5*-expression necessary for terminal differentiation (see Sect.

3.2.1), result in a domination of the latter (as no proliferation is found).

On the background of this balanced "potential to proliferate" of normal plasma cells, it is not surprising that aberrations in signaling or components of the cell cycle machinery can lead to (in the beginning slow) accumulation of plasma cells.

3.4.2 D-Type Cyclin Expression in Myeloma

Changes in signal transduction chains can lead to an increased (e.g., c-myc – CCND2) or aberrant CCND-expression, as can be mediated directly due to chromosomal aberrations at the cyclin-loci (e.g., translocation t(11;14) – aberrant expression of CCND1). An over or aberrant expression of CCND, frequent in malignant diseases (Sherr 1996; Sherr and Roberts 2004), is a hallmark of multiple myeloma (Bergsagel and Kuehl 2003). Compared to normal bone marrow plasma cells, almost all myeloma cells show a higher expression of at least one of the CCND. About half of the myeloma patients show an overexpression of CCND2 (expressed in bone marrow plasma cells) the other half an aberrant expression of CCND1 (not expressed in bone marrow plasma cells or cells of the B cell lineage). Aberrant expression of CCND3 is rare (<5% of myeloma patients). Aberrant expression can be caused by direct mechanisms: translocations involving the 11q13-locus and the heavy chain (IgH)-locus, 14q32, i.e., a t(11;14) leading to high CCND1-expression, rarely of light chain genes (t(2;11), t(11;22)). Linked to hyperdiploidy, gains of 11q13 lead to an aberrant CCND1-expression (lower compared to the one by t(11;14)). CCND3-expression (at least high) is mediated by a t(6;14) translocation involving the CCND3-locus at 6p21. In contrast, CCND2-overexpression is mostly indirectly mediated, i.e., by alterations in the signal transduction chain (e.g., t(4;14); aberrant FGFR3-expression).

CCND exemplify the general concept that different molecular alterations converge onto the same oncogenic pathways.

3.4.3 Proliferation of Malignant Plasma Cells

Despite a general CCND (over)expression (Bergsagel and Kuehl 2003; Hose et al. 2004, 2005) malignant plasma mostly show only a low proliferation rate (Drewinko et al. 1981; see Fig. 3.6). This rate increases from MGUS-patients over newly diagnosed and relapsed patients (Witzig et al. 1999; Bergsagel et al. 2005; Hose et al. 2011). Proliferation of malignant plasma cells is measured by various methods including 3H-thymidine uptake (Latreille et al. 1982; Boccadoro et al. 1984), Bromodeoxyuridine uptake (Schambeck et al. 1995; Lokhorst et al. 1986; Greipp et al. 1987), cell cycle analysis using propidium iodide, percentage of Ki67-expressing myeloma cells (Alexandrakis et al. 2004), and gene expression–based proliferation indices based on selected genes (Rosenwald et al. 2003; Bergsagel et al. 2005; Zhan et al. 2006). An example of the latter is the index by Shaughnessy et al. using the normalized expression-values of 11 genes associated with proliferation (*TOP2A*, *BIRC5*, *CCNB2*, *NEK2*, *ANAPC7*, *STK6*, *BUB1*, *CDC2*, *C10orf3*, *ASPM*, and *CDCA1*) scaled to the maximum within 22 normal bone marrow plasma cell samples (proliferation index of bone marrow plasma cells defined as 1; Zhan et al. 2006). Bergsagel et al. used the median of 12 genes associated with proliferation (*TYMS*, *TK1*, *CCNB1*, *MKI67*, *KIAA101*, *KIAA0186*, *CKS1B*, *TOP2A*, *UBE2C*, *ZWINT*, *TRIP13*, and *KIF11*) scaled to the maximum values over all samples (Bergsagel et al. 2005). Our group

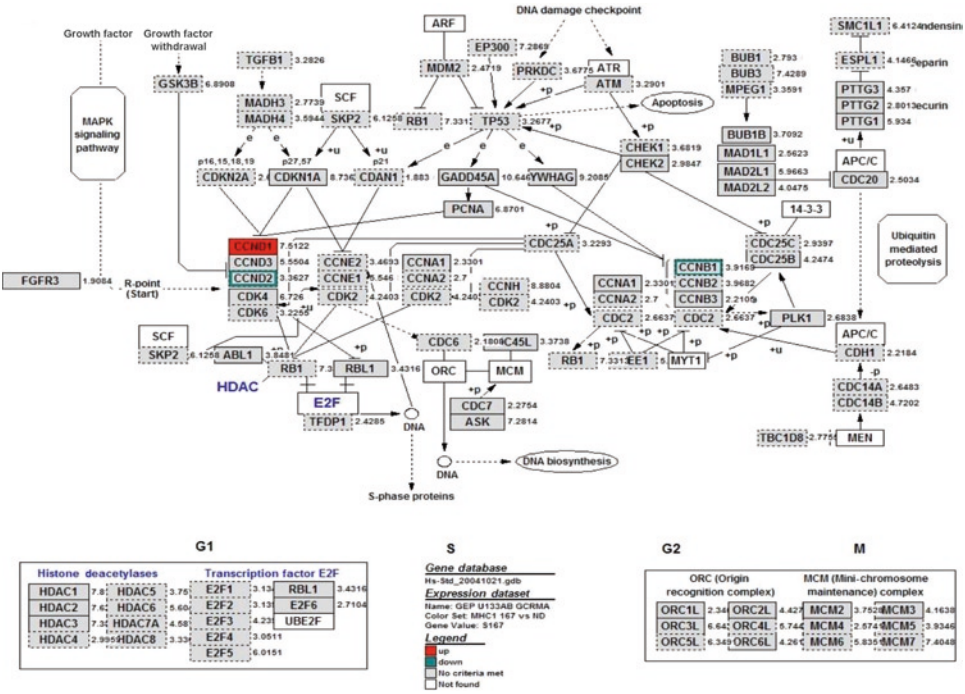


Fig. 3.6 *Cell cycle analysis*. Depicted is the core cell cycle machine for a particular patient (S167/02) relative to the median expression of the respective gene in seven bone marrow plasma cell (BMPC) samples. The patient harbors a hyperdiploid karyotype, gain of 11q13 without the presence of a translocation t(11;14), and an aberrant CCND1 expression (i.e., overexpression compared to BMPCs in which CCND1 is not expressed). In terms of molecular classification, the patient is attributed to EC1.1, TC 4p16, and Hy (hyperdiploid) in the molecular classification (see Sect. 3.4.1). Overexpressed genes are depicted in *red* (e.g., CCND1), under-expressed in *green*. A *green border* depicts down-regulation if a gene is represented by more than one probeset (here CCND2 is down-regulated for one probeset compared to BMPCs). *Grey* implies no differential expression. Structures not encoded by a single gene (e.g., APC) are depicted in *white*. Note that this myeloma cell sample shows a relatively unaltered cell cycle

proposed a gene expression–based proliferation index consisting of 50 genes (Hose et al. 2011). Proliferation of malignant plasma cells as assessed by different methods appears as strong prognostic factors in several analyses (Boccadoro et al. 1984; Greipp et al. 1988; San Miguel et al. 1995; Greipp et al. 1993; Gastinne et al. 2007; Zhan et al. 2006; Shaughnessy et al. 2007), independent of conventional prognostic factors, e.g., beta-2-microglobulin (Greipp et al. 1993), ISS, or presence of translocation t(4;14) (Hose et al. 2011).

3.5 Myeloma Cell Survival and Proliferation Factors

Numerous studies have been devoted to the identification of myeloma cell growth factors and to the signaling pathways leading to survival and/or proliferation of myeloma cells. A first category of factors activates the PI-3 kinase/AKT and MAP kinase pathways (IGF-1, insulin,

EGF family, HGF). A second category activates the JAK/STAT and MAP kinase pathways (IL-6, IFNα, IL-10, IL-21) and a third category the NF-kappa B pathways (BAFF/APRIL, TNF). See Fig. 3.3.

3.5.1 Interferon Alpha/Interleukin-6 Family and Activation of the JAK/STAT and MAP Kinase Pathways

IL-6 binds to a specific receptor (IL-6R) and the complex IL-6/IL-6R binds and induces the homodimerization of the gp130 IL-6 transducer (Heinrich et al. 2003). A remarkable feature of IL-6R is that its soluble form (sIL-6R) is an agonist molecule. It binds IL-6 with the same affinity as membrane IL-6R and the complex IL-6/sIL-6R binds and activates gp130 (Heinrich et al. 2003). The evidences of a major role of IL-6 in the survival and proliferation of malignant plasma cells are accumulated since the initial reports by others and us 14 years ago (Klein et al. 1989; Kawano et al. 1988). These evidences are the following:

1. Antibodies to IL-6 block myeloma cell proliferation and reduce the number of myeloma cells in cultures of patients' bone marrow cells in vitro by 50% (Klein et al. 1989; Zhang et al. 1992).
2. Injection of anti-IL-6 mAb inhibited myeloma cell proliferation in patients with terminal disease (Klein et al. 1991; Bataille et al. 1995) if the antibody was injected at a sufficient concentration to block the large IL-6 production in vivo (Lu et al. 1995a).
3. Serum levels of IL-6 and soluble IL-6R are increased in patients with multiple myeloma in association with a poor prognosis (Bataille et al. 1989; Gaillard et al. 1993).
4. IL-6 is overproduced by the bone marrow environment of patients with multiple myeloma, mainly by monocytes, myeloid cells, and stromal cells (Klein et al. 1989; Portier et al. 1991; Mahtouk et al. 2010). This production of IL-6 by the tumor environment is mostly mediated by IL-1 that is produced by monocytes and myeloma cells (Klein et al. 1989; Mahtouk et al. 2010; Costes et al. 1998). IL-1 induces PGE2 synthesis that further triggers IL-6 production (Costes et al. 1998). Thus inhibitors of IL-1 as the IL-1 receptor antagonists or of PGE2 synthesis might be interesting to block IL-6 production in patients with multiple myeloma. A similar mechanism was shown in the model of murine plasmacytoma in BALB/C mice. The generation of plasmacytomas was blocked by chronic administration of indomethacin that inhibited PGE2 synthesis and the large IL-6 production by the inflammatory environment (Hinson et al. 1996). Myeloma cells can also directly trigger IL-6 production by direct contact with bone marrow stromal cells by unidentified mechanisms (Uchiyama et al. 1993).
5. Cell lines whose survival is dependent on addition of exogenous IL-6 can be obtained from patients with extramedullary proliferation (Zhang et al. 1994a).
6. Mice transgenic with an IL-6 gene driven by the Eμ promoter develops massive polyclonal plasmacytosis (Suematsu et al. 1989). When crossed with murine BALB/c mice that spontaneously develop plasmacytomas, these crossed mice develop malignant plasma cells (Suematsu et al. 1992). In addition, knockout of IL-6 gene abrogated the generation of malignant plasmacytomas in BALB/C mice primed with mineral oil (Lattanzio et al. 1997).

Other cytokines of the IL-6 family are also myeloma cell growth factors due to the expression of specific receptors: OSM, CNTF, IL-11, LIF (Zhang et al. 1994b). But these factors are likely not involved in the emergence of the disease in vivo as they are weakly produced by the

tumor or its environment (Mahtouk et al. 2010). In our hands, we found that interferon-alpha (IFNα) is also a myeloma cell survival factor that is independent of IL-6 (Jourdan et al. 1991; Ferlin-Bezombes et al. 1998). IFNα activated the JAK/STAT and MAP kinase pathways as IL-6, in particular STAT3 phosphorylation (Lu et al. 1995a). Other groups found that IFNα could block myeloma cell proliferation. This discrepancy might be explained by the ability of IFNα to induce P19 inhibitor in some cell lines yielding to apoptosis (Arora and Jelinek 1998). Finally, IL-10 and IL-21 are also myeloma cell growth factors (Lu et al. 1995b; Menoret et al. 2008). IL-10 works through induction of autocrine loops of cytokines of the IL-6 family (Gu et al. 1996).

The myeloma cell survival activity of these cytokines is partly mediated by the phosphorylation of STAT3 by JAK kinases activated by the gp130 IL-6 transducer or IFN receptor. Blockade of JAK/STAT pathway by AG490 inhibits STAT3 phosphorylation and induces myeloma cell apoptosis (De Vos et al. 2000). STAT3 binding elements are found in the promoters of several anti-apoptotic proteins: MCL-1, bcl-2, bcl-xL. Among ten anti-apoptotic and pro-apoptotic proteins, we found that only MCL-1 was regulated by IL-6 or IFNα (Jourdan et al. 2000). Other groups suggested that bcl-xL was the main anti-apoptotic protein controlled by IL-6 in myeloma cells (Catlett-Falcone et al. 1999; Puthier et al. 1999), but a study emphasized that only a blockade of MCL-1, unlike bcl-2 or bcl-xL, could inhibit myeloma cell survival (Derenne et al. 2002). In addition, we found that induction of the constitutive production of MCL-1 by retroviral vector is sufficient to promote myeloma cell proliferation independently of IL-6 (Jourdan et al. 2003). IL-6 was reported to activate AKT kinase in myeloma cells that is able to trigger various signaling pathways (Tu et al. 2000). AKT activation can be mediated by STAT3 that can trigger PI-3 kinase activation (Pfeffer et al. 1997). In our experience, we found a weak AKT phosphorylation in only some IL-6-dependent cell lines. Actually, the IL-6-induced AKT phosphorylation in myeloma cells is weak and transient as compared to that induced by IL-6 (Mitsiades et al. 2002). PI-3 kinase-mediated AKT phosphorylation appears critical in promoting proliferation of myeloma cell lines since PI-3 kinase inhibitors abrogate it unlike MAP kinase inhibitors (Qiang et al. 2002; Pene et al. 2002).

3.5.2 Factors Activating the PI-3 and MAP Kinase Pathways: Insulin-Like Growth Factor 1, Heparin-Binding Growth Factors

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

IGF-1 plays likely a major role in myeloma in vivo. It is a survival and proliferation factor for most myeloma cell lines and primary myeloma cells (Georgii-Hemming et al. 1996; Jelinek et al. 1997). The reason is that IGF-1 receptor (IGF-1R) is aberrantly expressed by myeloma cells in association with poor prognosis (Sprynski et al. 2009). Indeed, IGF-1R is not expressed by normal plasma cells generated in vitro or in vivo. The reason for aberrant IGF-1R expression on myeloma cells is not known.

Large amount of IGF-1 are present in the bone marrow from patients (Hose et al. 2009a). First, IGF-1 gene is induced in the process of B to plasma cell differentiation and is also highly expressed by malignant plasma cells (Mahtouk et al. 2010). IGF-1 is also produced by osteoclasts (Mahtouk et al. 2010). Large amount of IGF-1 circulate in the blood in the form of a trimeric complex with IGF-BP3 and acid labile subunit in healthy individuals. IGF-1 plasma levels are not increased in patients with

multiple myeloma but are predictive of a poor survival (Standal et al. 2002). The biology of IGF-1 is complex since several IGF-binding proteins, mostly IGF-BP3, circulate at high concentrations and neutralize IGF-1 (Duan 2002). Cells may also express IGF-binding proteins that contribute to the biological activity of IGF-1 and disrupt the circulating IGF/IGF-BP complexes (Mahtouk et al. 2010). Myeloma cells also highly express the proteoglycan syndecan-1 (CD138) and can thus bind these trimeric complexes through IGF-BP3 (Beattie et al. 2005). This results in a weakening of the acid labile subunit binding and release of IGF-1 at the cell membrane of myeloma cells. Thus, IGF-1R is aberrantly expressed by myeloma cells, which produced IGF-1 and are bathed in vivo in large concentrations of IGF-1.

Regarding the transduction pathways, IGF-1 activates mainly PI-3 kinase pathway and in particular the phosphorylation of AKT protein (Sprynski et al. 2009; Ge and Rudikoff 2000) and its effect is independent of an activation of the JAK/STAT pathway (Jelinek et al. 1997; Ferlin et al. 2000). IGF-1 also induces MAP kinase phosphorylation (Sprynski et al. 2009; Ge and Rudikoff 2000). An inhibitor of PI-3 kinase pathway unlike a MAP kinase inhibitor (Qiang et al. 2002; Sprynski et al. 2009) blocks the myeloma growth factor activity of IGF-1. One mechanism of action of AKT is the phosphorylation of the pro-apoptotic protein Bad that induces its sequestration by the 14-13-3 protein and prevents its migration to mitochondrial membrane (Ge and Rudikoff 2000). The PI-3 kinase/AKT pathway in myeloma cells phosphorylates other proteins: the P70S6-kinase, forkhead proteins, and the glycogen synthase kinase-3 beta (GSK3b; Qiang et al. 2002; Pene et al. 2002; Hideshima et al. 2001). Phosphorylation of these proteins contributes to blockade of apoptosis and activation of cell cycle in various models. In particular, IGF-1 induces CCND1 and Skp2 expression and down-regulation of P27kip1 in myeloma cells (Pene et al. 2002). In addition, it was shown in one myeloma cell line that the PI-3 kinase/AKT pathway may activate the NF-kappa B pathway and expression of several targets of NF-kappa B involved in cell survival: A1/Bfl1, cIAP2, XIAP, survivin, FLIP (Mitsiades et al. 2002).

Transfection of myeloma cells with an activated AKT enhances tumor growth and protects from dexamethasone-induced apoptosis and expression of AKT dominant negative results in inhibition of IL-6-induced proliferation of myeloma cells (Hsu et al. 2002). The importance of the PI-3 kinase/AKT pathways for the survival and proliferation of myeloma cells is emphasized by deletion/mutation of the PTEN gene in some myeloma cells (Ge and Rudikoff 2000). PTEN is a phosphatase inhibiting the PI-3 kinase/AKT pathway and its deletion results in a high activation of PI-3 K/AKT pathway.

3.5.2.2 Insulin

Insulin and IGF-1 as well as their receptors are closely related molecules but both factors bind to the receptor of the other one with a weak affinity. Large levels of insulin are available in the blood plasma, produced by pancreatic beta cells in response to glucose level. The role of insulin in multiple myeloma was poorly studied. We have shown that insulin receptor (INSR) is increased throughout normal plasma cell differentiation (Sprynski et al. 2009). The *INSR* gene is also expressed by myeloma cells of newly diagnosed patients. Insulin is a myeloma cell growth factor as potent as IGF-1 at physiological concentrations and requires the presence of insulin/IGF-1 hybrid receptors, stimulating $INSR^{+}IGF\text{-}1R^{+}$ myeloma cells, unlike $INSR^{+}IGF\text{-}1R^{-}$ or $INSR^{-}IGF\text{-}1R^{-}$ myeloma cells (Sprynski et al. 2009). Immunoprecipitation

experiments indicated that INSR is linked with IGF-1R in myeloma cells and that insulin induced both IGF-1R and INSR phosphorylation and vice versa. Further therapeutic strategies targeting the IGF-IGF-1R pathway have to take into account neutralizing the IGF-1R-mediated insulin myeloma cell growth factor activity.

3.5.3 Heparin-Binding Factors

A hallmark of plasma cell differentiation is the expression of the proteoglycan syndecan-1 (CD138; Wijdenes et al. 1996; Costes et al. 1999). This heparan-sulfate protein has many biological activities and in particular is able to bind heparin-binding growth factors and present them to their specific receptors (Sanderson and Yang 2008). Thus, it is not surprising that several myeloma cell growth factors are heparin-binding molecules. Antibodies against CD138 are used for myeloma cell purification in clinical routine.

3.5.3.1 Heparin-Binding Epidermal Growth Factors

Using Atlas microarrays, we initially found that myeloma cell lines overexpress HB-EGF gene compared to EBV-transformed B cell lines or normal plasmablastic cells and that inhibitors of HB-EGF can block the IL-6-dependent survival of these myeloma cell lines (De Vos et al. 2001). Actually, we found that myeloma cells can bind large levels of EGF family molecules through heparan-sulfate chain of syndecan-1 molecules (Mahtouk et al. 2006). Myeloma cells express the four receptors of EGF family, ErbB1 through ErbB4. ErbB1 and ErbB2 are also expressed by normal plasma cells while ErbB3 and ErbB4 are aberrantly expressed by myeloma cells (Mahtouk et al. 2005). EGF members trigger the PI-3 kinase/AKT and MAPK pathways in myeloma cells, unlike STAT3 phosphorylation (Mahtouk et al. 2004). An inhibitor of the tyrosine kinase activity of these receptors can kill myeloma cells as well as primary myeloma cells (Mahtouk et al. 2004). We have also found that the EGF family members cooperate with IL-6 to trigger an optimal survival of myeloma cells, likely through an interaction between the transducer chains, gp130, and EGF receptors (Wang et al. 2002). These data indicate that ErbB inhibitors can potentiate dexamethasone-induced apoptosis of myeloma cell lines and of primary myeloma cells of most patients and suggest that they might improve treatment of patients with multiple myeloma.

3.5.3.2 Hepatocyte Growth Factor (HGF)

A study has shown that HGF is also a growth factor for myeloma cell lines (Derksen et al. 2002). HGF activity is blocked by removal of heparan-sulfate chains of syndecan-1 with heparitinase. This result indicates that syndecan-1 is critical to capture heparin-binding HGF and to present it to its receptor, cMet. Whether HGF cooperates with IL-6 to trigger myeloma cell survival was not investigated. Noteworthy, the XG-1 cell line used in this study was initially obtained in our laboratory and produces a low amount of autocrine IL-6 (Jourdan et al. 2005) that is sufficient to induce the HB-EGF activity. HGF is likely involved in the biology of myeloma. Indeed, HGF is expressed by 75% of myeloma cell samples, its serum level is increased and it is a prognostic factor in patients with multiple myeloma (Seidel et al. 1998). As HGF increases bone resorption, it may also be involved in the abnormal osteoclast resorption in patients with multiple myeloma (Hjertner et al. 1999).

3.5.3.3 Fibroblast Growth Factor (FGF)

A role of FGF in myeloma is suggested by the finding of a t(4;14) translocation affecting the FGF receptor type 3 in 15% of patients with multiple myeloma (Avet-Loiseau et al. 1998) (see Sect. 3.3.5). FGFs likely play an important role in myeloma biology because they bind syndecan-1 as HB-EGF or HGF and activation of FGFR3 induces the PI-3 kinase/AKT pathway that is critical for myeloma cell survival and proliferation.

3.5.4 Factors Activating NF-Kappa B: BAFF Family

BAFF and APRIL belong to the TNF family and activate at least three receptors of the TNF receptor family: BAFF-R, BCMA, and TACI. BAFF proteins are critical for the survival of B cells and may be involved in systematic lupus erythematosus. Activation of BAFF receptor family results in triggering the NF-kappa B pathway and likely other unidentified pathways (Mackay and Schneider 2009). Using DNA microarray or flow cytometric analysis, we and others found myeloma cells to express the two BAFF receptors, BCMA and TACI (Moreaux et al. 2004, 2009; Novak et al. 2004). BAFF-R is rarely expressed by myeloma cells (Moreaux et al. 2009). This observation prompted us to look for a role of the BAFF/APRIL in the survival/proliferation of myeloma cells. We found that two BAFF family proteins, BAFF or APRIL, are potent survival and proliferation factors of myeloma cells, depending on their expression of BAFF-R or TACI. In addition, BAFF or APRIL can protect myeloma cells from dexamethasone-induced apoptosis (Moreaux et al. 2004). Only a part of human myeloma cell lines do express TACI (Moreaux et al. 2007). As for primary myeloma cells, the $TACI^+$ myeloma cells have a mature plasma cell gene expression profiling (Moreaux et al. 2005). The results prompted us to perform a phase I trial with a BAFF/APRIL inhibitor, a TACI receptor fused with Fc fragment of human immunoglobulin (Rossi et al. 2009). TACI-Fc is a dimer. We observed a lack of toxicity of the treatment, a decrease in the concentration of polyclonal immunoglobulins in some patients 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 (Rossi et al. 2009).

3.5.5 Hierarchy of Myeloma Cell Growth Factors and Potential Clinical Applications

In the end, a minimum amount of growth factors need to be present in conjunction with chromosomal aberrations (see Sect. 3.3) to overcome the cell cycle break present in normal plasma cells (see Sect. 3.5). Some of the different components seem to be interchangeable, to a certain degree. High intrinsic CCND-expression (e.g., CCND1 as present in t(11;14)) might reduce the dependence on extrinsic growth factor stimulation. As reviewed above, several growth factors of myeloma cells have been documented, in particular because they are also critical for the generation of normal plasma cells: IL-6, IL-10, IL-21, IFNα, BAFF, and APRIL.

An exception is IGF-1 whose receptor is aberrantly expressed by about 50% of primary myeloma cells of newly diagnosed patients in association with a poor prognosis and 90% of myeloma cell lines (Sprynski et al. 2009). This aberrant IGF-1R expression confers a major myeloma cell growth activity to IGF-1 but also to insulin, both molecules being abundant in vivo.

In agreement with this pathophysiological observation, we and others have found IGF-1 being the major growth factor for myeloma cells, the effect of other growth factors being 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 (Menoret et al. 2008; Sprynski et al. 2009). The effect of IGF-1 is dependent on the expression of CD45 by myeloma cells. Indeed, the phosphatase CD45 can dephosphorylate and inactivate IGF-1R, conferring an important role for IL-6 to trigger the growth of $CD45^+$ myeloma cells (Descamps et al. 2006).

Another major point is the role played by syndecan-1 in myeloma biology. Syndecan-1 with three heparan-sulfate chains and two chondroitin-sulfate ones is mandatory for human myeloma cell growth in animal models. Targeting syndecan-1 or the heparan-sulfate chain synthesis blocks myeloma cell growth in vivo (Reijmers et al. 2010). Syndecan-1 may bind large amounts of growth factors (Mahtouk et al. 2006) and mobilize them close to growth factor receptors. This is likely the case for IGF-1, which circulates at a large concentration in the form of an inactive complex that can be disrupted by binding to syndecan-1.

Clinical implications of these findings are that targeting IGF-1R should be of major interest. One has to be aware of using inhibitors blocking both IGF-1 activation of IGF-1R homodimeric receptors and also insulin activation of IGF-1R/INSR hybrid receptors. IL-6 inhibitors should be also of major interest. These growth factor inhibitors have not to be used alone, since at the stop of the treatment, resumption of tumor growth will occur. This was observed in patients treated with anti-IL-6 antibodies. Inhibitors of myeloma cell growth factors have to be used in combination with cytotoxic agents as melphalan, dexamethasone, or bortezomib. Indeed, these growth factors can increase the resistance of myeloma cells to these drugs in vivo. In particular, we have documented the rise of large amounts of IL-6 9 days after high-dose melphalan in vivo (Condomines et al. 2010). This huge concentration of IL-6 will facilitate melphalan-resistant myeloma cells to repair their lesions in vivo. We have performed a phase II trial with anti-IL-6 antibody in association with high-dose melphalan (Rossi et al. 2005). This trial has shown the lack of toxicity of blocking IL-6 throughout high-dose melphalan and stem cell transplantation. It has also shown that patients treated with high-dose melphalan, stem transplantation, and anti-IL-6 had a survival advantage when mixed with a large series of matched patients treated with melphalan and stem cell transplantation alone (Rossi et al. 2005). In addition, drugs targeting efficiently the heparan-sulfate chains of syndecan-1, highly expressed by myeloma cells, will inhibit the biological effect of the majority of myeloma cell growth factors.

3.6 Multiple Myeloma Cells and the Microenvironment

Multiple myeloma is characterized by a progressive accumulation of myeloma cells within the bone marrow and a concomitant transformation of the bone marrow microenvironment. Hallmarks of the transformation process in the bone marrow are development of bone disease, impaired cellular immunity, and (increased) bone marrow angiogenesis (Chap. 4). We discuss in the following in depth the reciprocal interaction of myeloma cells and bone turnover as an example.

3.6.1 Pathogenesis of Myeloma-Induced Bone Disease

As normal plasma cells, myeloma cells are in tight *bidirectional* interaction with other cellular populations of the microenvironment as well as the extracellular matrix (Nagasawa 2006; Yaccoby et al. 2004; Abe et al. 2004). On the one hand, the bone marrow microenvironment forms a niche influencing plasma and myeloma cells being essential for their survival: Growth and survival factors like APRIL or IGF-1 are produced by osteoclasts (Moreaux et al. 2004; Sprynski et al. 2009), or, like IGF-1, liberated when bone-matrix is degraded during bone turnover. Additionally, a direct, e.g., integrin-mediated, interaction with fibronectin within the bone-matrix takes place (Shain et al. 2009; Tai et al. 2003). Furthermore, osteoclasts stimulate myeloma cell survival and proliferation via direct interaction (Yaccoby et al. 2004; Abe et al. 2004), especially involving $\alpha_4\beta_1$-integrin (Mori et al. 2004). On the other hand, myeloma cells influence the bone marrow microenvironment by increasing the number and activity of osteoclasts while reducing number and activity of osteoblasts, and destroying the three-dimensional structure of the bone remodeling compartment (BRC; see Fig. 3.7).

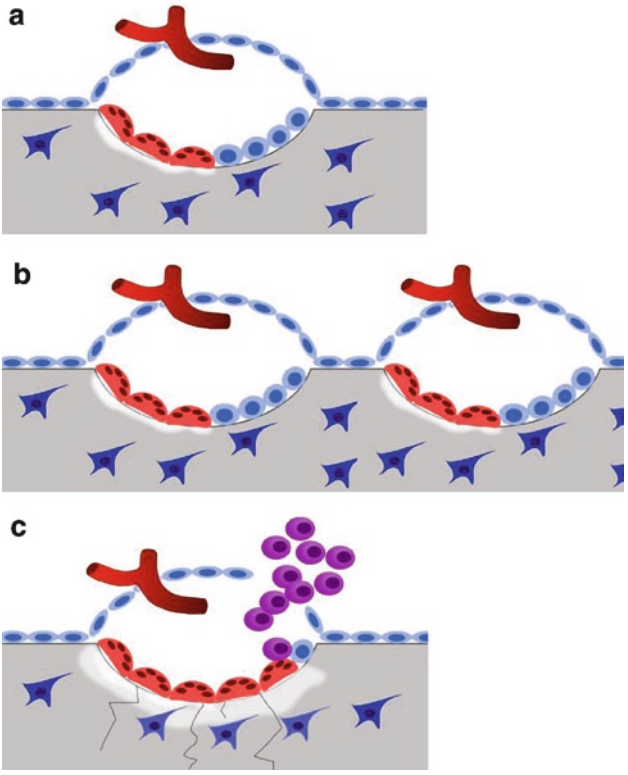


Fig. 3.7 *Myeloma-induced bone defects*. (**a**) Physiological situation. Bone formation by osteoblasts (*light blue*) and bone resorption by osteoclasts (*red*) are coupled. (**b**) In multiple myeloma, initially a higher bone resorption is found while bone formation keeps the pace (intact "bone remodeling compartments", BRCs). (**c**) If BRCs are disrupted due to interaction with myeloma cells (*violet*), bone resorption is increased and bone formation almost completely abrogated

(1) *Increase in osteoclast number and activity*: Normal and malignant plasma cells produce osteoclast-activating or osteoclast-generating mediators like vascular endothelial growth factor A (VEGFA; (Hose et al. 2009a). In a co-culture model of osteoclasts and myeloma cells, a simultaneous inhibition of VEGF and osteopontin inhibits angiogenesis and bone resorption almost completely (Tanaka et al. 2007). In vitro, VEGF can substitute the stimulating effect of macrophage-colony stimulating factor (M-CSF) on differentiation of osteoclasts (Niida et al. 1999). Further factors are macrophage inflammatory proteins (MIP)-1α and MIP-1β (Terpos et al. 2003a), which directly increase production rate and resorption activity of osteoclasts by binding to the receptors CCR1 and CCR5 (Oba et al. 2005). At the same time, they increase expression of receptor activator nuclear factor kappa B ligand (RANKL)- and IL-6 expression by bone marrow stromal cells and indirectly stimulate osteoclasts (Abe et al. 2002; Oba et al. 2005; see below). Furthermore, myeloma cells shift the OPG:RANKL-ratio on osteoblasts by aberrant expression of Wnt-signaling inhibitors like dickkopf 1 (DKK1; Tian et al. 2003) or secreted frizzled related protein-2 (sFRP-2; Oshima et al. 2005). Physiologically, DKK1 is produced by bone marrow stromal cells and osteoblasts. DKK1 inhibits Wnt3A-signaling via LRP5/6 leading to a consecutive shift in the OPG:RANKL-expression on osteoblasts in favor of RANKL. Osteoprotegerin (OPG) likewise produced by osteoblasts and bone marrow stromal cells is, as soluble decoy-receptor for RANKL, its physiological antagonist (Simonet et al. 1997). OPG-secretion by bone marrow stromal cells and osteoblasts is reduced after direct cellular interaction with myeloma cells (Pearse et al. 2001; Giuliani et al. 2001). Compared to healthy individuals, myeloma patients show increased RANKL- and decreased OPG-serum levels (Pearse et al. 2001; Giuliani et al. 2001; Politou et al. 2004). Increasing serum-RANKL:OPG-ratios correlate with extent of disease and survival (Terpos et al. 2003b). Whether RANKL is also expressed by primary myeloma cells or myeloma cell lines is discussed controversially (Sezer et al. 2002; Giuliani et al. 2001, 2002; Yaccoby et al. 2007; Haaber et al. 2008). Increased RANKL-expression by osteoblasts and bone marrow stromal cells (Pearse et al. 2001) is a central feature. Interaction with receptor activator of nuclear factor-κB (RANK) on osteoclast-precursors and osteoclasts stimulates production and resorption activity of osteoclasts (Lacey et al. 1998).

(2) *Reducing the number of osteoblasts*: Myeloma cells express functional inhibitors of the differentiation from mesenchymal stromal (stem) cells to osteoblasts. An example is HGF. HGF is expressed by malignant plasma cells of about 60% of myeloma patients (Standal et al. 2007; Hose et al. 2009a). High serum-HGF-level correlate here negatively with the serum level of bone-specific alkaline phosphatase (as marker of osteoblast activity; Standal et al. 2007). In vitro, HGF inhibits BMP-induced osteoblastogenesis from mesenchymal stromal cells (Standal et al. 2007). It lifts the BMP-induced arrest of proliferation of mesenchymal stromal cells necessary for differentiation. A direct cell-to-cell interaction between myeloma cells and bone marrow stromal cells leads to increased IL-6 and RANKL-production whereas OPG-production is concomitantly reduced (Giuliani et al. 2001; Shipman and Croucher 2003), in turn again stimulating osteoclastogenesis.

(3) *(Self-)limiting interaction*: We and others have shown recently that normal as well as malignant plasma cells produce factors stimulating osteoblast differentiation and activity, e.g., BMP6 (Seckinger et al. 2009) or adrenomedullin (Cornish et al. 1997; see Sect. 3.2.2). This could be eventually interpreted as self-limitation of the impact of plasma and myeloma cells on bone turnover, in analogy to osteoblasts, which likewise produce RANKL *and* OPG.

Taken together, myeloma cells have the ability to induce a reduced number of osteoblasts with a RANKL:OPG-ratio shifted to RANKL (osteoclastogenesis), and an increased number and activity of osteoclasts (see Fig. 3.7). To understand the in vivo situation, however, the microanatomical structure of bone remodeling and interaction with myeloma cells needs to be understood.

(4) *Role of intact "bone remodeling compartments"*: Histomorphometric investigations report myeloma patients to show an increase in number and activity of osteoclasts (Valentin-Opran et al. 1982; Taube et al. 1992; Bataille et al. 1991). The number of osteoblasts in early stages of myeloma is likewise increased, but decreases over time together with osteoblast activity in patients with bone lesions (see Fig. 3.7; Bataille et al. 1990, 1991; Giuliani et al. 2005; Standal et al. 2007). Andersen et al. published recently a very insightful analysis of the role of the BRCs and an intact canopy of osteoblast like cells on the magnitude of bone resorption/formation activities (Andersen et al. 2009, 2010). They compared the extent of erosion and osteoid surfaces (1) in control bone, (2) in myeloma biopsies showing more than 75% of the total erosion under intact BRC canopies (MM-I), and (3) in those with at least 75% erosion under disrupted BRC canopies (MM-D). MM-I biopsies show increased erosion surface, osteoclast surface, and osteoid surface compared to controls. MM-D biopsies show even more increased erosion surface and osteoclast surface compared to MM-I biopsies, but in contrast, their osteoid surface falls below control levels, thereby indicating lack of bone formation despite increased bone resorption. In control and MM-I biopsies, increased osteoid surface parallels increased erosion surface, indicating coupling between bone formation and resorption. In contrast, in MM-D biopsies, erosion surface increases strongly without corresponding increase in osteoid surface, indicating absence of coupling between bone formation and resorption. Thus, bone formation responds commensurately to bone resorption only when the BRC canopy is continuous. The same conclusion holds true if the analysis is based on osteoclast surface and if the myeloma biopsies are grouped according to the proportion of osteoclast surface in intact BRCs. Bone formation occurs very preferentially in intact BRCs as also seen when analyzing the proportion of osteoid in intact BRCs; this proportion averages 75% in all three groups of biopsies, despite their differences in overall extent of osteoid surface. This is in marked contrast with erosion, which proceeds whether BRCs are intact or not and becomes even higher in the latter case. The authors deduced a close link between the integrity of BRC canopies and the magnitude of osteoclast and osteoblast activities. In summary, if BRCs are disrupted, bone resorption tends to increase and bone formation to be prevented (Andersen et al. 2009, 2010), whereas in intact BRCs present in controls, MM-I, and patients with hyperparathyroidism, bone formation increases with bone resorption (Andersen et al. 2009; Hauge et al. 2001).

3.6.2 Patterns and Healing of Bone Defects

Nothing is currently known about causes of different *patterns of bone defects* in multiple myeloma, e.g., diffuse and focal patterns. Healing of bone defects, if present, appears also in patients with complete remission at orders of magnitude slower as compared to the healing of fractures (Epstein and Walker 2006), comparable with the delayed healing of osteoporotic fractures; likewise, the reason remains unclear. Possible scenarios are the presence of remaining residual myeloma cells, which maintain a continuous stimulation of bone resorption vs. bone formation (Esteve and Roodman 2007), a loss of the stimulus to repair bone defects, and a "scorched earth" left over by destroyed BRCs

and pathological remodeling in bone defects. At the same time, the bone marrow microenvironment might remember former presence of myeloma cells over years. Evidence is given by in vitro differentiated osteoblasts from myeloma patients, which show a different expression pattern compared to those differentiated from normal donors (Corre et al. 2007).

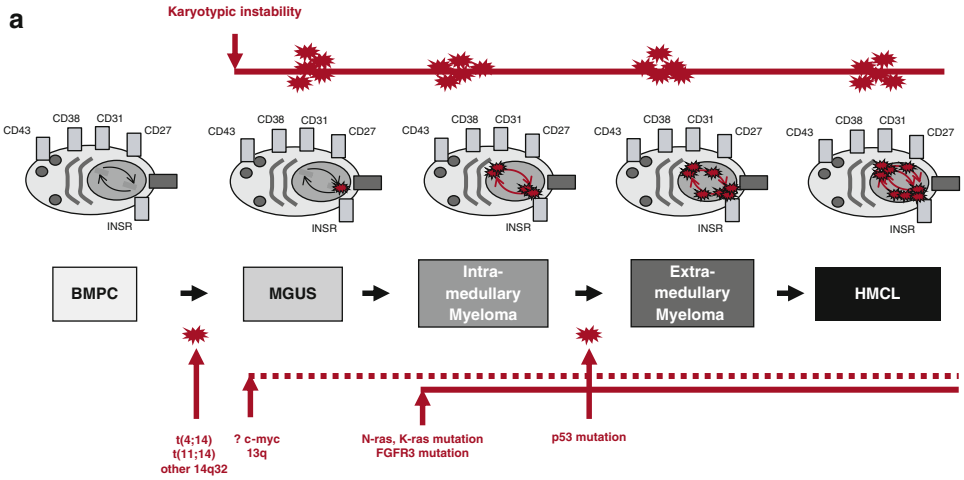
3.6.3 Therapeutic Strategies for Treatment and Prevention of Myeloma Bone Disease

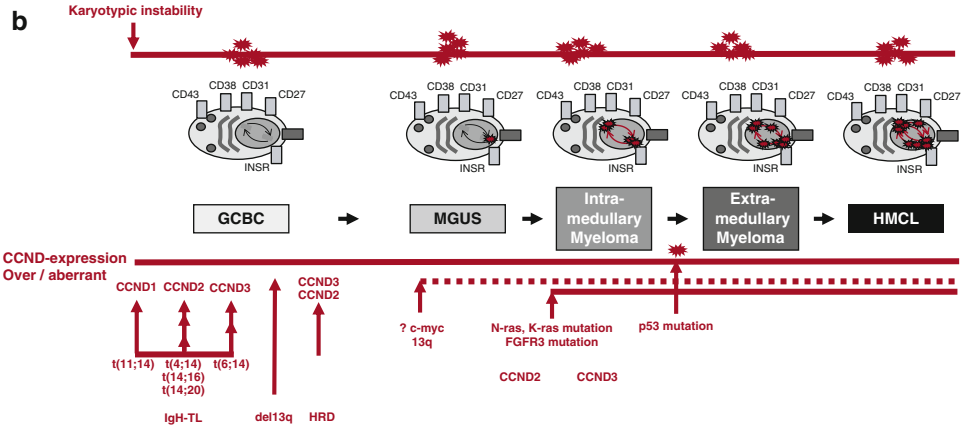
Amino-bisphosphonates like zoledronate induce apoptosis in osteoclasts (Kellinsalmi et al. 2005) and significantly reduce skeletal events in patients with malignant bone destruction (Rosen et al. 2004). Amino-bisphosphonates show – albeit limited – activity against myeloma cells (Aviles et al. 2007). RANKL-antibodies like denosumab show direct inhibition of osteoclastogenesis (Lewiecki 2006). Novel agents used in myeloma treatment like proteasome inhibitors (bortezomib) or IMiDs (lenalidomide) exhibit at systemic application besides their activity against malignant plasma cells an impact on osteoblast and osteoclast function. Lenalidomide inhibits resorption by osteoclasts, but seems not to influence osteoblast function (Breitkreutz et al. 2008; De et al. 2009). Bortezomib induces apoptosis in myeloma cells (Richardson et al. 2005), inhibits bone resorption by osteoclasts (von Metzler et al. 2007), and stimulates osteoblast activity (Heider et al. 2006). The latter is of special interest; as with parathyroid hormone, only one bone-anabolic compound is approved for systemic application. For local use, BMP2 and BMP7 are approved (Gautschi et al. 2007; Tsuji et al. 2006).

An appropriate functionalization of biomaterials using pathophysiological knowledge for local treatment of bone defects in multiple myeloma especially with bone formation promoting agents seems thus to be a promising approach.

3.7 Pathogenetic Model of Multiple Myeloma

We will conclude this chapter with some more general reflections on factors influencing myeloma cell accumulation and a proposal for a new pathogenetic model of multiple myeloma (Fig. 3.8).




b
Karyotypic instability
CD43 CD38 CD31 CD27
INSR
GCBC
MGUS
Intra-medullary Myeloma
Extra-medullary Myeloma
HMCL
CCND-expression
Over / aberrant
CCND1 CCND2 CCND3
CCND3
CCND2
t(11;14) t(4;14) t(6;14)
t(14;16)
t(14;20)
IgH-TL
del13q
HRD
? c-myc
13q
N-ras, K-ras mutation
FGFR3 mutation
p53 mutation
CCND2
CCND3

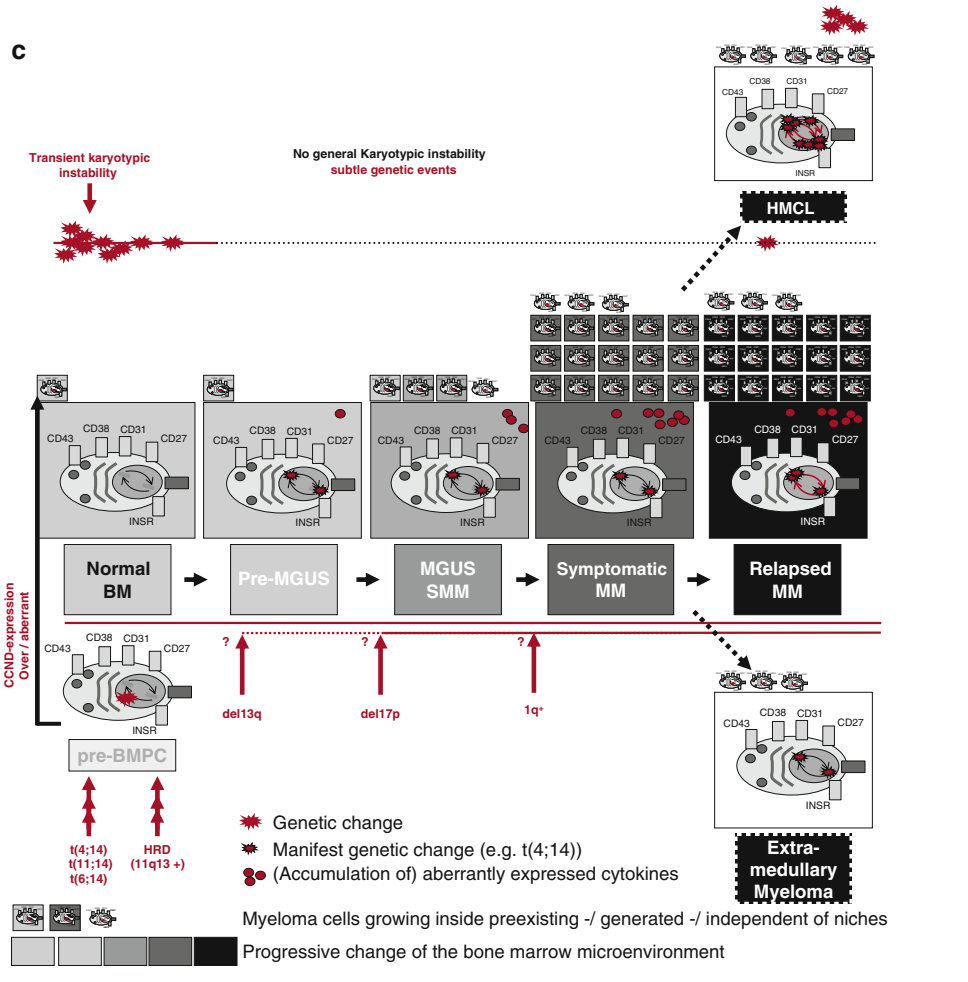

c
Transient karyotypic instability
No general Karyotypic instability
subtle genetic events
HMCL
Normal BM
Pre-MGUS
MGUS SMM
Symptomatic MM
Relapsed MM
CCND-expression
Over / aberrant
del13q
del17p
1q+
pre-BMPC
t(4;14)
t(11;14)
t(6;14)
HRD
(11q13 +)
Extra-medullary Myeloma
Genetic change
Manifest genetic change (e.g. t(4;14))
(Accumulation of) aberrantly expressed cytokines
Myeloma cells growing inside preexisting -/ generated -/ independent of niches
Progressive change of the bone marrow microenvironment

Fig. 3.8 *Models of pathogenesis of multiple myeloma*. The models of Hallek et al. 1998(A) and Bergsagel et al. 2005(B) focus on a sequel of genetic aberrations driving changes of gene expression on (malignant) plasma cells that in turn lead to a transformation of the bone marrow microenvironment (BMME). Our model (C) proposes the accumulation of hijacked "normal" plasma cells accumulating in the bone marrow and thus initially driving changes in the bone marrow microenvironment. (**a**) Model from Hallek et al. 1998. The model proposes an ongoing karyotypic instability (indicated by *red stars*) starting at MGUS-stage and leads to multiple accumulating genetic lesion (*red stars* with *black border*). Bone marrow plasma cells (BMPCs) or precursors are targeted by recurrent IgH-translocations. Plasma cells progress from a premalignant MGUS-stage in a sequel from intramedullary to extramedullary myeloma with human myeloma cell lines (HMCLs) being the end stage. Each step of this sequel is driven by an additional genetic event. Dysregulation of c-myc is thought to appear early, ras-mutation and eventually mutations of FGFR3 appear beginning with the intramedullary myeloma-stage. p53 mutations appear as late event. (**b**) The model from Bergsagel et al. (2005) focuses on the earliest oncogenic changes that are thought to involve three overlapping pathways and occur in germinal center B cells (GCBC). They are present in MGUS thought to be premalignant tumors. Two partially overlapping pathways, indicated by IgH-translocations and multiple trisomies, generate non-hyperdiploid and hyperdiploid tumors, respectively. A third pathway (del13q) leading to monosomy of chromosome 13 or deletion of 13q14 can be present in both types of tumors, but occurs with a higher prevalence in non-hyperdiploid tumors, where it occurs in almost all tumors with t(4;14) and t(14;16), but infrequently in tumors with t(11;14). The essentially invariant dysregulation of a CCND (aberrant/overexpression) is associated with these early oncogenic changes. Recurrent IgH-translocations and the dysregulation of CCND are used to group MGUS and myeloma according to the TC-classification (see Sect. 3.4.1). (**c**) Proposed new model. Two principal pathways targeting plasma cell precursors (pre-BMPCs), most likely post-germinal-center B cells, i.e., translocations most often involving the IgH-locus, and a hyperdiploid pathway. Both lead to increased CCND-expression, overexpression (CCND2) or aberrant expression (CCND1, CCND3). Karyotypic instability is in place only at this time (indicated by *red stars*). Targeted pre-BMPCs home to the normal plasma cell niche (indicated by a *grey box*). The BMME (*light-grey box*) is unaltered. These cells already have a slightly dysregulated cell cycle (hijacked "normal" plasma cells) and the tendency to accumulate (see text for details). In pre-MGUS-stage, the transformation process of the BMME begins slowly. Initially, pre-MGUS cells share the niche with BMPCs. A further accumulation leads to MGUS/smoldering MM (SMM) stage without the necessity of further genetic events. The BMME is slowly transformed by normal BMPC-factors (indicated by the increasingly *dark grey*) and aberrantly expressed factors (*red dots*). Aberrant expression is driven mainly by the changing microenvironment, not accumulating genetic alterations. Malignant plasma cells populate existing BMPC-niches (*light-grey boxes*), recruit new niches (*dark grey boxes*) and partially gain independence from the BMME (plasma cell without a box). Further accumulation of malignant plasma cells leads to therapy-requiring myeloma. The BMME transformation continues (*darkening grey*, increased number of aberrantly expressed factors) in a positive feedback loop. A further selection pressure to recruit new niches and grow independently of niches is in place. HMCLs can be derived from therapy-requiring or relapsed myeloma, i.e., cells that already gained partial independence of the BMME. They do represent a further step of myeloma development. The same holds true for extramedullary myeloma that does not regularly appear, even in end-stage patients. Progression-related aberrations (del17p, 1q21 gain) can appear with increasing frequency throughout accumulation of malignant plasma cells; these aberrations appear with a certain probability and are thus more frequent in relapsed myeloma; at least $1q21^{+}$. For detailed discussion, see Sect. 3.8

3.7.1 Disease Activity, Tumor Load, and Molecular Characteristics of Myeloma Cells

3.7.1.1 Describing Disease Activity

Main determinants of *disease activity* at a *given time* are the *tumor-load* (total number of plasma cells) and *molecular characteristics* of myeloma cells. Tumor-load and molecular characteristics are to a certain degree independent at a given time (e.g., an aggressive lesion can be present together with high and low tumor mass), but interdependent, if the time course is taken into account (an aggressive lesion will lead faster to a higher tumor mass and might have, e.g., a higher bone turnover stimulating capacity).

Molecular characteristics at a given time represent a flash image of (1) myelomagenesis (etiology), (2) entity (e.g., HRD/non-HRD, t(4;14)-myeloma, GEP-based group), and (3) accumulated evolutionary (progression-related) aberrations (e.g., gain of 1q21, loss of p53-expression). Whereas as a matter of definition etiologic aberrations cannot change over time, for the disease entity this depends on the definition of the latter. iFISH-based entities (e.g., t(4;14), HRD myeloma) seem to be constant throughout the course of myeloma. This likewise holds true for GEP-based groups with the exception of the proliferation group within the molecular classification (Zhan et al. 2006) to which patient-attributed other groups can progress, e.g., patients from MS (t(4;14)) at diagnosis to PRL in relapse.

Molecular characteristics comprise a further important feature of myeloma cells: their "biological activity", e.g., potential to generate bone lesions, induce angiogenesis, or immunosuppression (e.g., expression of cancer testis antigens; Condomines et al. 2007, 2009 or CD200; Barclay et al. 2002; Moreaux et al. 2006). This biological activity is not necessarily connected to disease etiology or entity as exemplified by the promotion of bone disease by DKK1-expression.

The *total number of plasma cells* is mediated by five main variables: (1) The proliferation rate, i.e., speed of cell division; (2) the survival (or death-) rate, comprising of (a) apoptosis rate ("suicide") and (b) (T-)cell mediated elimination-rate ("killing," a host factor), the first two taken together as growth rate, (3) the dissemination rate, i.e., the ability of myeloma cells to spread to different bone marrow parts and niches therein, (4) the rate of transforming the bone marrow microenvironment and thus the creation of additional niches, and (5) the rate of gaining independence of niches. Regarding the latter factors, whereas normal bone marrow plasma cells depend on *extrinsic* survival signals provided within a special niche, myeloma cells can gain a certain independence of these by autocrine production (e.g., IL-6), induction of the production in the bone marrow microenvironment, e.g., IL-6 via amphiregulin produced by myeloma cells, and recruitment of factors abundant in serum (e.g., IGF-1) by expression of respective receptors (Sprynski et al. 2009; see Fig. 3.8). An additional, less understood, mechanism is an *intrinsic* loss of dependence on these factors, e.g., by aberrant CCND1-expression due to presence of a t(11;14) mimicking a respective growth factor stimulation converging on the G_0/G_1-transition. Human myeloma cell lines, carrying a plethora of chromosomal aberrations and being only dependent on serum factors in their culture medium, are a special example. These variables are partially interdependent, e.g., transformation of the bone marrow microenvironment and recruitment of additional survival factors can influence apoptosis rate. Over time, the proliferation *(growth) rate* becomes a very important feature, also transmitting to prognostic significance (see Sect. 3.5).

A further important characteristic of myeloma cells within one patient is their potential intrapatient-*heterogeneity*. Evidence is given by the presence of subclonal-, and the emergence of "progression-related" aberrations like gains

of 1q21 (see Sect. 3.3). It is therefore an interesting question whether the two possibilities of myeloma cell accumulation – generation of niches and obtaining the ability to grow independently of these – take part in the generation of intra-patient clonal heterogeneity. This heterogeneity might likewise be present in terms of a part of the myeloma cell population being "myeloma stem cells," a controversial discussion outside the scope of this chapter.

3.7.1.2 Interpatient Heterogeneity: Many and Multiple Myelomas

Discernable chromosomal aberrations (e.g., IgH-translocations vs. hyperdiploidy) and a plethora of changes in gene expression are present in different multiple myeloma patients, i.e., a huge molecular interpatient heterogeneity. Clinically, multiple myeloma is on the one hand a rather homogeneous disease, with plasma cell accumulation in the bone marrow, and almost all patients developing increased bone marrow angiogenesis and bone lesions. On the other hand, multiple myeloma is very heterogeneous in terms of survival (see Sects. 3.3.5 and 3.4.2). As discussed above, on a molecular level, almost all patients show the presence of either an IgH-translocation or a hyperdiploidy driven pathway, and almost all show a CCND dysregulation. This notion has lead to the idea of "many and multiple myelomas" (Fonseca 2003).

Thus, the same clinical phenotype (e.g., accumulation of plasma cells, induction of bone disease and angiogenesis) can be reached by different molecular phenotypes, i.e., different alterations of DNA and gene expression. For example, there has been up to now no single unifying aberration or change in gene expression found explaining bone disease or angiogenesis in myeloma (Hose et al. 2009a).

From a theoretical point of view, there are two possible explanations: (1) Targets of aberrations converge on a limited number of intermediary molecules of signal transduction ("*molecular hubs*"). If a certain intermediary is needed to be activated for myeloma cell survival/proliferation or a specific feature of myeloma cells like induction of bone disease, selection pressure could lead on different ways to this necessary alteration. If, e.g., increased ras-signaling would be critical, this could be due to, e.g., (a) autocrine IL-6 production, (b) increased IL-6 production in the bone marrow microenvironment by AREG-expression of myeloma cells, and (c) IGF-1 expression via ras/MAPK signal transduction, or constitutive ras-activation in myeloma (Klein et al. 2003; Neri et al. 1989; Liu et al. 1996). Another example is given by *D-type cyclin* expression – the hallmark of multiple myeloma – which can be due to several molecular causes (see Sect. 3.5). Here, CCND could exemplify a final integrator of signal transduction by external (growth factor stimulation) and internal (aberrant CCND-expression) signals. (2) Myeloma cells are "just hijacked" normal plasma cells in terms of an initially (subtle) takeover of cell cycle control leading to a slow induction of accumulation of plasma cells but otherwise use of (physiological) plasma cell features explaining clinical features of myeloma. This could easily explain why different aberrations targeting cell cycle and especially CCND lead to the same clinical phenotype of multiple myeloma (see also the following). According to this model, a low number of aberrations targeting the cell cycle takes place very early in the development of myeloma, i.e., in post-germinal center B cells. The accumulation of plasma cell-like myeloma cells, i.e., hijacked "normal" plasma cells, then changes the bone marrow microenvironment. Not investigated up to now, expression changes in myeloma cells could be attributed to epigenetic changes driven by the changing bone marrow microenvironment, not primary genetic events.

3.7.2 Multistep Transformation of Myeloma Cell Model

This model initially described by Hallek et al. (Hallek et al. 1998; Fig. 3.8a) is based on a proposed *sequel of progressive genetic* events that profoundly change the pathophysiological features of myeloma cells at each step and then lead to the ordered progression from a normal plasma cell to MGUS, where the cells are immortalized, but not transformed, and do not progressively accumulate or cause bone destruction; to intramedullary myeloma, where the cells are confined to the bone marrow microenvironment, accumulate and cause bone destruction; to extramedullary myeloma, where the cells proliferate more rapidly and grow in the blood (plasma cell leukemia) or other extramedullary sites; and to a myeloma cell line, where the cells may be propagated in vitro. Critical oncogenic events in myeloma cells are thought either to occur after or do not interfere with most of the normal differentiation process involved in generating a long-lived plasma cell. The model evokes a karyotypic instability thought to appear in MGUS and continues throughout all stages of tumor progression, giving rise to the different molecular events in relation to clinical progression. 14q32-translocations are seen as a potential early event, concordant with isotype switch recombination, so that it precedes MGUS. Some translocations (e.g., t(11;14)) were thought to lead more rapidly to fulminant disease, eventually bypassing an MGUS-stage. For other aberrations, the timing was not clear but nonetheless thought to be in some kind of 7der, including monosomy 13 or dysregulation of c-myc. In patients with aberrant FGFR3 expression caused by t(4;14), a mutation of FGFR3 could lead to ligand independence and clinical progression (Sibley et al. 2002). Mutations of N- and K-ras are not present in MGUS, but are present in intramedullary myeloma, with an increasing incidence as the disease progresses. Mutations of p53 are a late event associated with aggressive extramedullary myeloma.

Current additions are the presence of a presumed second pathway (i.e., hyperdiploid myeloma) independent of IgH-translocations (Fig. 3.6; Bergsagel and Kuehl 2005; Bergsagel et al. 2005; Fig. 3.8b).

This model basically focuses (1) on the genetic changes within myeloma cells (i.e., genetic alterations causing aberrant expression) as driving force for myeloma cell progression and *concomitantly* for changes within the bone marrow microenvironment; the changes within the bone marrow microenvironment are thus driven by the "malignant features" of malignant plasma cells; and (2) on an underlying broad chromosomal instability as a driving force.

As of now, parts of this concept need to be reevaluated: First, there is currently only evidence for rather subtle changes, and an ongoing genetic instability has never been proven with nonproliferation-dependant methods (see Sect. 3.3 and below). Second, several features attributed to myeloma cells are already such of normal plasma cells, including the ability to induce angiogenesis (see Sect. 3.8.3.1). As mentioned before, part of the change within the bone marrow microenvironment could be driven by accumulation of "semi-normal" plasma cell–like myeloma cells. "Semi-normal" here refers to this change being due to normal plasma cell features in cells "hijacked" to limited proliferation. Third, the proposed sequel from normal plasma cells, MGUS, intramedullary multiple myeloma, extramedullary and cell line–like myeloma seems to be rather an exception than the rule. Extramedullary myeloma is a special feature in a subpopulation of patients, and eventually, even in these, a subpopulation of myeloma cells. Myeloma cell lines are only obtainable in less than 10% of patients and almost never in hyperdiploid multiple myeloma (Fig. 3.6).

3.7.3 Transformation of Bone Marrow Microenvironment Model

3.7.3.1 Features of Normal Plasma Cells as Explanation for Those of Myeloma Cells

Capabilities of malignant plasma cells are partly explainable by physiological functions of their normal counterpart, bone marrow plasma cells. The primary feature of the latter is being antibody-production facilities. Evidence is given that they re-structure their surroundings (bone marrow microenvironment) according to their needs: (1) by securing their own supply by a basal angiogenic stimulus, e.g., the production of VEGFA (Hose et al. 2009a); (2) bone marrow plasma cells can interact with the microenvironment and bone remodeling by production of factors like BMP6 (see Sect. 3.2.2; Seckinger et al. 2009); (3) connected to this or via an independent process, bone marrow plasma cells are able to *create to a certain extent their survival niche*. The niche is critical to allow normal plasma cells surviving for several years (see Sect. 3.2.1). The number of survival niches is thought to be limited and thus help in maintaining a fairly constant number of plasma cells over life, evidenced by a constant level of polyclonal immunoglobulin, despite the ability of the immune system to adapt to novel antigen challenges, and thus the creation of novel plasma cells that have to compete for a survival niche; thus, "niching" per se is a dynamic process. Furthermore, bone marrow plasma cells can create niches under certain conditions, as exemplified in cases of reactive plasmacytosis, in which their number can increase for a prolonged amount of time. Much less is known about the ability of bone marrow plasma cells to interact with the hematopoietic and the immune system. Taken together, molecular alterations in the pre-bone marrow plasma cell are according to our concept a crucial factor for molecular pathogenesis of myeloma, as the proliferation arrest is removed and the "potential to proliferate" liberated, leading to accumulation of hijacked "normal" plasma cells, which by itself generates changes in the bone marrow microenvironment without the a priori need for enforced selection of myeloma cell variants with additional aberrations.

3.7.3.2 Pre-MGUS-Stage

At this stage, founder cells (myeloma cells) are present, but the "disease" activity is far below the (detection) limit defining "MGUS," even by molecular techniques. Initial *etiological chromosomal aberrations* (probably related to a hyperdiploid and a non-hyperdiploid pathway, see Sect. 3.3.2) lead to subtle cell cycle alteration (direct or indirect CCND over or aberrant expression). Cell cycle breaks are initially unaltered leading to a very low proliferation rate with doubling times of months or even years. T cell–mediated elimination of aberrant cells is intact. The apoptosis rate is comparable to the one of normal plasma cells. These cells presumably populate the same survival niche as normal bone marrow plasma cells allowing their longevity. Pre-MGUS myeloma cells are basically hijacked "normal" plasma cells.

3.7.3.3 MGUS-Stage/Smoldering Myeloma

Continuing accumulation of myeloma cells in the bone marrow leads to a detectable but asymptomatic "disease" – MGUS. It is now clear that MGUS consistently precedes myeloma (Landgren et al. 2009). The accumulation slowly transforms the bone marrow microenvironment,

initially mainly by factors already produced by normal bone marrow plasma cells (e.g., VEGFA). The total production of these factors is increased due to the increasing number of hijacked "normal" plasma cells/myeloma cells. At the same time, a selection pressure for myeloma cells is in place due to the limited number of niches – either to create new niches comparable to those of normal bone marrow plasma cells, or gain a certain independence by recruiting new sources of growth and survival factors, i.e., by aberrantly producing such factors (e.g., HGF, amphiregulin, IL-6) or by inducing their production within the bone marrow microenvironment (e.g., IL-6) to increase the availability of growth factors present in serum (e.g., IGF-1 by better blood vessel supply (angiogenesis)), access to growth factors for which myeloma cells carry receptors, but the bone marrow microenvironment does not express ligands (e.g., FGF:FGFR3). A further tappable source of growth and survival factors is the alteration of bone turnover. Initially, BRCs are relatively intact (see Sect. 3.7) and the surrounding bone marrow unaltered. Some leaking out of these complexes (e.g., of IGF-1 liberated from bone-matrix) is likely. An increased bone turnover would lead to an increase of total leaking despite BRCs being intact. At a later stage, largely increased liberation will appear as a consequence of disrupted BRCs, leading in turn to lytic bone lesions (see Sect. 3.7). Interaction of myeloma cells with the BRCs (osteoblasts and osteoclasts) is presumably initially mostly driven by factors already expressed by normal plasma cells, e.g., BMP6. Nevertheless, myeloma cells aberrantly express such factors as exemplified by the Wnt-antagonist DKK1 (Li et al. 2006). We hypothesize these factors to be already expressed at the earliest stage, in agreement with complete lack of evidence of an appearance only in disease progression.

3.7.3.4 Symptomatic Myeloma

Further accumulation of myeloma cells leads to an increasing concentration of plasma cell and aberrantly expressed myeloma cell growth factors. As mentioned above, this aberrant expression is not necessarily the consequence of genetic alteration but could also be driven by the changing bone marrow microenvironment, in turn leading to expression changes within hijacked "normal" plasma cells driving these to an increasingly abnormal expression pattern. This could explain the plethora of expression changes (see also Sect. 3.4) without the prerequisite of an ongoing genetic instability. The factors act together in terms of a positive feedback mechanism: better growth conditions lead to an increased speed of accumulation of myeloma cells and in turn a better adaption of the bone marrow microenvironment according to the need of myeloma cells. The increasing transforms of the bone marrow microenvironment become clinically visible in terms of (1) increased angiogenesis, (2) bone destruction (breakup of BRCs and subsequent generation of osteolysis and generalized osteopenia), (3) reduced tumor surveillance, and (4) increased plasma cell infiltration based on generation additional survival niches for myeloma cells. As mentioned above, we hypothesize that accumulation of plasma cell-like myeloma cells could already explain a basic appearance of these features without the a priori necessity of directed extensive molecular changes within the myeloma cells (see above). This could also elegantly explain the lack of one myeloma typical aberration. Several aberrations ultimately converge on hubs and at least in part on CCND (see Sect. 3.8.1). A positive feedback loop would be a good explanation for a scenario of relatively long slow growth by creating additional niches with a subsequent "outbreak" of therapy-requiring myeloma once an additional source is tapped (as a BRC).

The possible explanation that pathogenetic features of myeloma are driven by cell cycle targeted "hijacking" of normal plasma cell functions is of fundamental interest, as it takes away the necessity of the requirement of accumulation of further chromosomal aberrations for progression within the sequel from MGUS to overt myeloma and plasma cell leukemia as proposed in the model of Hallek et al. (see Sect. 3.8.2; Hallek et al. 1998). The proposed principal role of plasma cell accumulation notwithstanding, a likely subtle selection pressure may be present in terms of factors promoting (faster) plasma cell accumulation (Sect. 3.8.1.1). To this end, growth factor stimulation substantially present due to the changing bone marrow microenvironment might lead to an increased tendency to proliferate and "overrule" cell cycle checkpoints inhibiting growth of cells, in particular with chromosomal aberrations, again in a positive feedback mechanism. Secondary chromosomal aberrations (e.g., del17p, loss of p53) and mutations (e.g., ras) would further increase independence of cell cycle checkpoints. It has to be emphasized that this seems to be a rather subtle process, not the presence of an *ongoing* and widespread chromosomal instability. This nonewithstanding, there seems to have been at a certain (early) time during myelomagenesis for a set period such an instability, explaining the plethora of chromosomal aberrations, but again, it could not be taken as proven explanation for the disease progression from early MGUS to smoldering and therapy-requiring myeloma. As depicted in Sect. 3.3.1, only metaphase (proliferation dependent) cytogenetics show a prominent increase in the number of chromosomal aberrations with disease progression (Jonveaux and Berger 1992) and are therefore not representative for the presence of chromosomal aberrations. This increase has indeed up to now not been verified by (proliferation independent) iFISH data on large cohorts of patients. Further investigations including next generation sequencing will show whether this relative stableness is also present if a genome-wide screen for mutations is performed. If it holds true that the main features of myeloma cells might be already in place during pre-MGUS-stage as a consequence of "hijacked" normal plasma cells, another consequence would be that our perception of "monoclonal gammopathy of unknown significance" might change, and, speculatively, the last two words eventually will be dropped (see Fig. 3.8c).

We would like to finish this chapter with an urban myth – the attributed blessing, or curse, of a Chinese philosopher for a newborn – to live in interesting times. Whatever the true origin, this has surely become true for myeloma research – in a positive sense.

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