

# Understanding the hypoxic niche of multiple myeloma: therapeutic implications and contributions of mouse models

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Multiple myeloma (MM) is the second most common hematological malignancy and is characterized by the clonal expansion of plasma cells in the bone marrow. Recently, hypoxia has received increased interest in the context of MM, in both basic and translational research. In this review, we describe the discovery of the hypoxic niche in MM and how it can be targeted therapeutically. We also discuss mouse models that closely mimic human MM, highlighting those that allow preclinical research into new therapies that exploit the hypoxic niche in MM.

## Introduction

Multiple myeloma (MM) represents a fatal B-cell malignancy that is characterized by the monoclonal proliferation of malignant plasma cells (PCs) in the bone marrow (BM). MM accounts for approximately 10% of all hematological malignancies and represents 1-2% of all cancer-related deaths. The diagnosis of MM is based on the presence of 10% or more clonal PCs in the BM or the presence of a biopsy-proven plasmacytoma, plus evidence of end-stage organ damage (e.g. anemia, hypercalcemia, lytic bone lesions or renal failure) attributable to the underlying PC disorder (Kyle and Rajkumar, 2004; Kyle and Rajkumar, 2008). Despite substantial progress in understanding disease pathogenesis and developing therapies, MM still remains a fatal disease, with a median survival of 7-8 years after therapy (Anderson, 2011).

MM cells reside in the BM, which has a well-organized architecture and is composed of many different types of stromal cells, including osteoblasts, osteoclasts, BM endothelial cells, fibroblasts and adipocytes. It has been demonstrated that the BM niche can aid the growth and spreading of MM cells by a complex interplay of cytokines, chemokines, adhesion molecules, proteolytic enzymes and other components of the extracellular matrix. All of these elements provide an optimal growth environment for MM and play essential roles in MM cell growth. Importantly, the BM microenvironment was shown to confer survival and

chemoresistance of MM cells to current therapies (Hu et al., 2010; Azab et al., 2012). Recent preclinical and early clinical studies indicate that new therapies that target not only MM cells but also their interactions with the host BM microenvironment might improve the outcome of MM treatment (Lemaire et al., 2011; Pagnucco et al., 2004; Ribatti et al., 2006; Hideshima et al., 2007; Basak et al., 2009). Preclinical *in vivo* models that recapitulate the natural behavior of MM and the clinical response to therapies are important prerequisites for the translation of investigational anti-MM therapies. In this Commentary, we discuss the hypoxic niche of BM that has been invaded by MM cells and review the mouse models that have been used in basic and preclinical studies of MM. We also give an example how hypoxia might be targeted in treating MM by drawing on evidence obtained using the mouse 5T33MM model.

## Hypoxia and MM

Hypoxia is an imbalance between oxygen (O<sub>2</sub>) supply and consumption that deprives cells or tissues of sufficient oxygen. Decreases in oxygen levels are observed in certain types of physiological processes and pathological situations; in both cases, hypoxia-inducible factors (HIFs) are key factors involved in hypoxia responses (Box 1). As described below, hypoxia has been well characterized in solid tumors, whereas its role in MM remains less clear.

## Hypoxia in solid tumors: significance and effects on malignant progression

Unlike in normal organs, the partial pressure of O<sub>2</sub> (pO<sub>2</sub>) in solid tumors shows a range of median O<sub>2</sub> tensions from 1.3-3.9% (10-30 mmHg), with readings recorded as low as 0.01% (0.08 mmHg). Importantly, 82% of all measurements taken in solid tumors were less than 0.33% O<sub>2</sub> (2.5 mmHg) (Höckel and Vaupel, 2001). Traditionally, hypoxia has been considered a marker of tumor progression, and has become a central issue in tumor physiology and treatment. An O<sub>2</sub> tension below 10 mmHg (corresponding to less than 1.3% O<sub>2</sub> in the atmosphere) was thus proposed as the hypoxia criteria *in vivo* (Höckel and Vaupel, 2001). Importantly, in solid tumors, it became clear that hypoxia can initiate tumor cell death by apoptosis or necrosis, but also prevent tumor cell death by provoking adaptive responses that, in turn, enhance tumor proliferation, angiogenesis and metastasis, thus contributing to the development of an aggressive phenotype. Moreover, independent of standard prognostic factors, tumor hypoxia has been suggested to be an adverse prognostic factor for patient outcome (Vaupel, 2009).

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**Box 1. HIFs and mechanisms of hypoxia sensing**

Of all of the proteins induced by hypoxic conditions, hypoxia-inducible factors (HIFs) are the major transcription factors and master regulators of adaptive responses to hypoxia (Pugh and Ratcliffe, 2003; Ruas and Poellinger, 2005). The HIFs are heterodimeric complexes composed of an inducible  $\alpha$ -subunit (HIF-1 $\alpha$ , HIF-2 $\alpha$  or HIF-3 $\alpha$ ) and a constitutively expressed  $\beta$ -subunit. Of the three HIF family members, HIF-1 and HIF-2 are the most well characterized and are the key regulators of oxygen homeostasis (Loboda et al., 2010; Prabhakar et al., 2009). The stability of HIF-1 $\alpha$  and HIF-2 $\alpha$  is O<sub>2</sub> sensitive. In the presence of O<sub>2</sub>, prolyl hydroxylases modify HIF-1 $\alpha$  and allow it to interact with the von Hippel Lindau (VHL) complex, thereby resulting in ubiquitylation and proteasome-dependent degradation (Semenza, 2001). Moreover, O<sub>2</sub>-dependent hydroxylation of HIF-1 $\alpha$  by the enzyme factor inhibiting HIF (FIH) will block the interaction of HIF-1 $\alpha$  with the transcriptional coactivator p300-CBP and therefore inhibit HIF-1 $\alpha$ -mediated gene transcription in normoxia (Jeong et al., 2002). In hypoxic conditions, the rate of asparagine and proline hydroxylation decreases, VHL can no longer bind to HIF-1 $\alpha$  and the ubiquitylation of HIF-1 $\alpha$  is inhibited, thus resulting in an accumulation of HIF-1 $\alpha$ . The stabilized HIF-1 $\alpha$  then translocates to the nucleus, where it interacts with HIF-1 $\beta$  and p300-CBP to activate transcription of target genes mediating adaptive cellular responses to hypoxia (Ogura et al., 2002; Semenza, 2002).

**The O<sub>2</sub> extension level in the BM niche: normoxic or hypoxic?**

In contrast to other organs, the BM has long been accepted to be a naturally hypoxic organ (Pennathur-Das and Levitt, 1987; Danet et al., 2003). However, data from different researchers were initially confusing. By using a cannula inserted into the femoral long bones of anesthetized dogs, Tøndevold et al. reported that the pO<sub>2</sub> values in the epiphyseal and the diaphyseal regions were from 25.2 mmHg (3.32%) to 8.1 mmHg (1.07%) and from 26.7 mmHg (3.51%) to 8.3 mmHg (1.09%), respectively (Tøndevold et al., 1979). In the metaphyseal region, the pO<sub>2</sub> values were from 18.9 mmHg (2.49%) to 10.9 mmHg (1.43%). The mean values of intraosseous pressure measured by Tøndevold et al. were between 20 and 30 mmHg (2.63–3.95%). A study in 82 men with chronic bronchitis showed that the mean pO<sub>2</sub> in the BM was 48 mmHg [6.32%; range 33.5–64.1 mmHg (4.41–8.43%)] (Skouby, 1976). In another study performed in five healthy volunteer BM donors, immediate assessment of oxygen levels in the BM aspirates showed an average O<sub>2</sub> tension of 54.9 mmHg (7.22%) (Harrison et al., 2002). Recently, another study performed in the BM aspirates of six healthy donors and 44 patients with MM or MGUS (monoclonal gammopathy of undetermined significance) further showed an average O<sub>2</sub> tension of 54 mmHg, which was still considered hypoxic (Colla et al., 2010). However, these studies do not take into account the fact that the O<sub>2</sub> spatial distribution in the BM is heterogeneous.

**Methodological challenges to measuring O<sub>2</sub> tension level in the BM**

Different groups have demonstrated that, when using the bioreductive hypoxia marker pimonidazole, the BM of mice has a heterogeneous O<sub>2</sub> distribution, with distinct BM compartments having different pO<sub>2</sub> levels (Lévesque et al., 2007; Parmar et al., 2007; Shin et al., 2008). In the BM, the incorporation of pimonidazole (which cross-links to a protein adduct at O<sub>2</sub> tension below 10 mmHg) was found to be markedly high along the

endosteum, together with high levels of HIF-1 $\alpha$  expression, whereas the central vascular niche was less hypoxic and had lower levels of HIF-1 $\alpha$  expression. The latter finding can be explained by the location of the central vascular niche, being close to the oxygenated blood. It has been suggested that this heterogeneous level of pO<sub>2</sub> in the BM contributes to the process of hematopoiesis. That is, the hypoxic endothelial niche maintains the hematopoietic stem cells in a typical 'stem cell' status characterized by immature and quiescent cells, and the vascular niche (with higher pO<sub>2</sub> levels) supports the maturation and proliferation of these stem cells (Wilson and Trumpp, 2006; Parmar et al., 2007; Martin et al., 2011).

In contrast to other organs and solid tumors, the anatomy of the BM, and especially the presence of the bone, is a major hurdle for noninvasive oxygen measurements. In addition, when using the invasive needle oxygen electrode techniques for detecting tissue pO<sub>2</sub> levels, one cannot show the heterogeneous distribution of O<sub>2</sub> tension in the BM. Therefore, in the BM, *ex vivo* methods including staining for exogenous or endogenous markers of hypoxia, such as pimonidazole and HIF-1 $\alpha$ , might be a better choice (Box 2).

**Roles of hypoxia in the pathophysiology of MM**

Although hypoxia has been demonstrated to be crucial for normal BM hematopoiesis, the role of hypoxia in the etiology, pathogenesis and possible treatment of hematological malignancies such as MM is still unknown (Lennon et al., 2001; Wilson and Trumpp, 2006; Parmar et al., 2007). Several groups have investigated hypoxia in human MM. Using immunohistochemistry staining of the endogenous hypoxia markers HIF-1 $\alpha$  and HIF-2 $\alpha$  in fixed bone biopsies obtained from MM patients, 35/106 (33%) cases demonstrated high level of HIF-1 $\alpha$  stabilization, and 14/106 (13.2%) cases had a high level of HIF-2 $\alpha$  expression (Giatromanolaki et al., 2010). Following these findings, two recent studies also confirmed the expression of HIF-1 $\alpha$  and HIF-2 $\alpha$  in BM biopsy specimens from MM patients (Martin et al., 2010; Colla et al., 2010). However, because these three studies of MM did not test the expression of HIF-1 $\alpha$  and HIF-2 $\alpha$  in normal BM, the difference in expression of these markers in normal versus MM BM was unclear. The study by Colla et al. further tested the O<sub>2</sub> tension in the BM of MM patients, MGUS patients and healthy donors by immediately measuring aspiration of BM samples with a gas analyzer (Colla et al., 2010). However, the median pO<sub>2</sub> and oxygen saturation (sO<sub>2</sub>) values measured in BM samples of patients with MM or MGUS were analogous to those of healthy volunteers, and values were not hypoxic when compared with the criteria of hypoxia in tumors [10 mmHg (1.3% O<sub>2</sub>)]. In addition, as mentioned earlier, the values of pO<sub>2</sub> in BM from different labs also show large discrepancies, which raise methodological questions regarding how to measure the pO<sub>2</sub> in BM; as noted in Box 2, no single method can test all aspects of BM.

Recently, we investigated and compared the difference in oxygen tension in the BM of naive and 5T33MM mice (see below for details of these mice) by assessing the exogenous and endogenous hypoxia markers pimonidazole and HIF-1 $\alpha$ , respectively (Hu et al., 2010). Our data clearly show that both the exogenous marker pimonidazole and the endogenous hypoxia marker HIF-1 $\alpha$  were dramatically increased in the BM of 5T33MM tumor-bearing mice. In contrast, the distribution of hypoxia markers in the normal BM is weak and sporadic, suggesting hypoxia is a specific feature of MM BM. This

was recently confirmed in the  $V\kappa^*$ MYC transgenic model (Chesi et al., 2012). Another study from our lab using the 5T2MM mouse model found that the degree of hypoxia in MM BM was less than that in normal BM. In this case, we measured the levels of accumulated pimonidazole in flushed BM samples using flow cytometry (Asosingh et al., 2005). We believe that these results with the 5T2MM model are inconsistent with those mentioned above because we chose a different method to measure hypoxia; this again highlights the importance of methodology (Box 2).

Given that hypoxia has a known role in solid tumor progression, it is reasonable to believe that the hypoxic niche also plays an essential role in MM biology. In solid tumors, hypoxia is known to drive angiogenesis; notably, the BM microenvironment in MM is also characterized by an increased microvessel density (Vacca et al., 1994). Studies of MM suggest that increased BM angiogenesis is due to the aberrant expression of angiogenic factors by MM cells and other cells in the microenvironment. It has been demonstrated that a pathway involving HIF, vascular endothelial growth factor (VEGF) and VEGF receptor is upregulated in ~40% of MM cases and is linked to increased angiogenesis in MM (Giatromanolaki et al., 2010). In addition, the tumor suppressor ING4 has been identified as a repressor of angiogenesis by suppressing HIF activity in MM (Colla et al., 2007). Furthermore, HIF-2 $\alpha$ -mediated upregulation of the chemokine ligand CXCL12 was demonstrated to contribute to aberrant angiogenesis in MM (Martin et al., 2010). As in other settings, HIFs are clearly central in MM hypoxia, and a recently published review by Martin et al. discusses the roles of HIFs in the pathogenesis of MM in-depth (Martin et al., 2011).

### Treatment of MM by targeting the hypoxic niche: clinical significance

Accumulating data on the biological aspects of tumor hypoxia suggest that hypoxia could be exploited for cancer therapy, and a growing number of compounds targeting hypoxia are being explored in preclinical and clinical development. To target tumor hypoxia in cancer treatment, two major strategies are being developed: (1) drugs that inhibit molecular targets in the hypoxia signaling pathway (e.g. HIF-1 $\alpha$  inhibitors) and (2) bioreductive prodrugs that are activated selectively by hypoxia (e.g. TH-302).

### Therapeutically targeting key molecules in the hypoxia signaling pathway

Studies investigating the molecular mechanisms that mediate cellular responses to hypoxia have increased knowledge of targets that might compromise the survival of hypoxic tumor cells. HIF-1 $\alpha$  is one well-defined target in both solid tumors and MM (Podar and Anderson, 2010; Semenza, 2003; Poon et al., 2009; Zhang et al., 2009). In MM, a number of anti-MM drugs, such as adaphostin (Zhang et al., 2009), lenalidomide (Lu et al., 2009), bortezomib (Shin et al., 2008) and 17-AAG (Mitsiades et al., 2006) have been shown to inhibit HIF-1 $\alpha$  activity. In addition, inhibition of HIF-1 $\alpha$  activity can increase melphalan-induced apoptosis in MM cells (Hu et al., 2009). Targeting HIF-1 $\alpha$  in hypoxic tumor cells with more specific small-molecule inhibitors – including YC-1 (Yeo et al., 2003), PX-478 (Welsh et al., 2004), EZN-2968 (Greenberger et al., 2008), Polyamide 2 (Olenyuk et al., 2004), NSC 50352 (Park et al., 2006) and Echinomycin (Kong et al., 2005) – is being developed and tested in different solid tumors.

### Box 2. Methods for measuring O<sub>2</sub> tension

Below we summarize the methods currently available and undergoing development for detection of hypoxia *in vivo*. There are many methods to detect tumor hypoxia, and the method most appropriate for a particular experiment depends on the nature of the approach with respect to (1) invasiveness, (2) the degree of resolution required and (3) whether measurement of a direct or indirect parameter is necessary. In solid tumors, the current 'gold standard' is intratumor polarographic measurement of O<sub>2</sub> partial pressures using microsensor techniques that adhere to the systematic random sampling principle. However, it is unlikely that a single method will be suitable for all situations; therefore, use of more than one technique is often advisable. In all instances, careful interpretation of the data obtained is paramount, and researchers should bear in mind the exact parameters measured and the limitations of the specific methods used.

#### (1) Invasive microsensor techniques

- Polarographic O<sub>2</sub> sensor (Höckel et al., 1991; Vaupel et al., 1991)
- Luminescence-based optical sensor (Collingridge et al., 1997; Griffiths and Robinson, 1999)

#### (2) Noninvasive detection of sensitizer adducts by positron emission tomography (PET) or single-photon emission computed tomography (SPECT)

- <sup>18</sup>F-fluoromisonidazol (<sup>18</sup>F-FMISO) (Oehler et al., 2011)
- <sup>18</sup>F-azomycin-arabioside (<sup>18</sup>F-FAZA) (Garcia-Parra et al., 2011)
- <sup>60</sup>Cu-ATSM (Lewis et al., 2008)
- <sup>18</sup>F-EF3 (Leung, 2004; Christian et al., 2007)
- <sup>18</sup>F-EF5 (Komar et al., 2008)
- <sup>123</sup>I-iodoazomycin-arabioside (Moore et al., 1993)
- <sup>99m</sup>Tc-HL91 (Cook et al., 1998)

#### (3) Techniques for intravascular O<sub>2</sub> detection

- Cryospectrophotometry (HbO<sub>2</sub> saturation) (Fenton et al., 1988)
- Near-infrared spectroscopy (HbO<sub>2</sub> saturation) (Hull et al., 1999)
- Phosphorescence imaging (Wilson and Cerniglia, 1992)

#### (4) Nuclear magnetic resonance (NMR) spectroscopy and imaging techniques

- <sup>1</sup>H NMR (Pacheco-Torres et al., 2011; Weljie et al., 2011)
- <sup>19</sup>F-magnetic resonance relaxometry (van der Sanden et al., 1999)
- Combretastatin A-4-phosphate (CA4P) (Zhao et al., 2011)

#### (5) Noninvasive electron paramagnetic resonance (EPR) oximetry

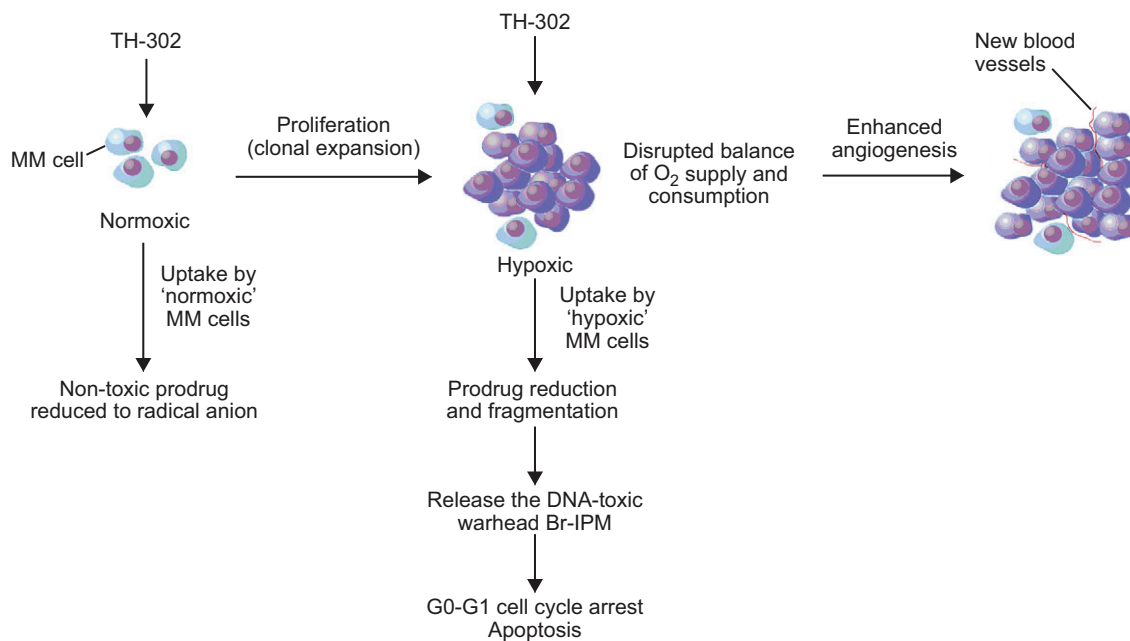
#### (6) Invasive immunohistochemistry (IHC) with exogenous hypoxia markers

- Misonidazole (<sup>3</sup>H-MISO) (Cline et al., 1997)
- Pimonidazole (PIMO) (Raleigh et al., 1996)
- Etanidazole (EF5) (Marotta et al., 2011)
- Nitroimidazole-theophylline (NITP) (Hodgkiss et al., 1997)

#### (7) Invasive IHC with endogenous hypoxia markers (Rademakers et al., 2011).

### Targeting MM with a hypoxia-activated prodrug

In solid tumors, tumor hypoxia has been considered a potential therapeutic problem because it renders the tumors more resistant to treatment. However, it is thought that tumor hypoxia can also be exploited for selective treatment with approaches such as those involving hypoxia-activated cytotoxins (Bennewith and Dedhar, 2011; Wilson and Hay, 2011). In a preclinical study performed in the 5T33MM mouse model (summarized in Fig. 1), we demonstrated that hypoxia-activated treatment with TH-302 as a monotherapy shows efficacy in MM (Hu et al., 2010). TH-302 is a 2-nitroimidazole-based nitrogen mustard prodrug. After the hypoxia-dependent release of its cytotoxic warhead [bromo-isophosphoramidate mustard (Br-IPM)], it acts as a DNA cross-



**Fig. 1. Targeting MM with the hypoxia-activated prodrug TH-302.** TH-302 is a potent and highly selective hypoxia-activated prodrug developed by Threshold Pharmaceuticals. It is a 2-nitroimidazole prodrug of the cytotoxin bromo-isophosphoramidate mustard that exhibits hypoxia-selective cytotoxicity across a broad spectrum of human cancer cell lines in vitro, and shows in vivo efficacy in a large panel of human tumor xenografts. During normal disease progression, MM cells exist in an increasingly hypoxic niche (purple cells), which enhances angiogenesis and promotes disease progression. The uptake and reduction of TH-302 by hypoxic MM cells triggers apoptosis and G0-G1 phase cell cycle arrest in a dose-dependent manner (Hu et al., 2010). TH-302 is also being evaluated in Phase 1/2 clinical trials for the treatment of solid tumors as a monotherapy and in combination with other chemotherapeutic agents.

linking agent to kill hypoxic tumor cells (Wilson and Hay, 2011). More recently, by combinational targeting of the hypoxic niche with TH-302 and the proteasome with bortezomib, we demonstrated that this combination synergistically induced apoptosis in MM cells (Hu et al., 2011). Importantly, the combination of TH-302 and bortezomib in the 5T33MM mouse model showed impressive improvements in multiple disease parameters and resulted in a significantly prolonged survival. This study provides novel insight into the potential application of bortezomib and hypoxia-activated treatment for MM, and provides support for clinical evaluation of this combination for MM patients (Hu et al., 2011). Currently, a phase 1/2 clinical trial of TH-302 as monotherapy or in combination with bortezomib in individuals with relapsed/refractory MM has been launched at the Dana Farber Cancer Institute of Harvard University (<http://clinicaltrials.gov/ct2/show/NCT01522872>).

Targeting hypoxia in other hematological cancers has also provided promising results. Recently, a preclinical trial using acute lymphoblastic leukemia (ALL) xenograft models to test PR-104, another hypoxia-activated prodrug, also showed high efficacy (Benito et al., 2011). Moreover, the results of a phase 1 clinical trial conducted at MD Anderson Cancer Center showed that TH-302 can reduce blasts in patients with advanced acute myelogenous leukemia (AML) or ALL. One patient had a complete response with incomplete platelet recovery, and resolution of leukemia cutis (<http://investor.thresholdpharm.com/releasedetail.cfm?ReleaseID=633096>; <http://www.clinicaltrials.gov/ct2/show/NCT01149915?term=th-302&rank=7>). These results indicate that targeting the hypoxic niche in leukemic BM using bioreductive hypoxia-activated prodrugs might represent a promising new option for leukemia

therapy. More information about the mechanism of action and development of bioreductive hypoxia-activated prodrugs can be found in a recently published review (Wilson and Hay, 2011).

### Mouse models of multiple myeloma

There is a consensus in the field that the lack of good animal models of MM hampers a full understanding of disease mechanisms and the successful translation of new MM therapies (Anderson, 2011; Taniguchi and Taniguchi, 1975; Yaccoby et al., 1998). The ideal animal model would reproduce all facets of human MM, but available models only resemble the disease in some aspects (DeWeerd, 2011). Appropriate use and improvement of animal models for MM will undoubtedly help to clarify the pathological mechanisms and benefit the development of treatment approaches (Table 1). In the remainder of this review, we discuss currently available mouse models of MM, with special emphasis on those models that can be used to study the role of hypoxia in MM pathology and treatment.

### The natural model of MM: 5TMM series

The 5TMM models are based on de novo spontaneous B-cell proliferative disorders that develop in elderly mice of the C57BL/KaLwRij strain (Radl et al., 1979). 5TMM models are propagated by in vivo transfer of myeloma cells from sick mice into young syngeneic recipients (Fig. 2). Several series of 5TMM models exist, but the 5T33MM and 5T2MM models are the best characterized and the most commonly used. Disease develops in the 5T33MM model in 3-4 weeks, whereas the disease develops in 12 weeks in the 5T2MM model. The latter also consistently develops a clear osteolytic bone disease, similar to the human disease

**Table 1. Comparison of different mouse models of MM**

Model	BM involvement	BM homing	Osteolytic bone lesion	Angiogenesis	Immuno-competent host	Syngeneic system	Applicable to hypoxia studies	Reference
5TMM series	Yes	Yes	Yes	Yes	Yes	Yes	Demonstrated	Asosingh et al., 2000; Vanderkerken et al., 2003
Conventional s.c. SCID xenograft	No	No	No	Yes	No	No	Not suitable owing to s.c. xenograft, not BM-related	Pilarski et al., 2000; Mitsiades et al., 2003
SCID-hu	Implanted bone, locally	No	Occasionally	Yes	No	Only within implanted bone	Could be investigated	Urashima et al., 1997; Yaccoby et al., 1998; Tassone et al., 2005
SCID-rabbit	Implanted bone, locally	No	Occasionally	Yes	No	No	Could be investigated	Yata and Yaccoby, 2004
SCID-synth-hu	Implanted bone, locally	No	No	Yes	No	Only within implanted scaffold	Could be investigated	Calimeri et al., 2011
Orthotopic SCID	Preferentially in axial skeleton	Yes	Locally in spine	Yes	No	No	Could be investigated	Mitsiades et al., 2003
RAG-2 knockout	Yes	Yes	Yes	Yes	No	Yes	Could be investigated	Fowler et al., 2009
Transgenic: E $\mu$ -c-MAF	Yes	?	Yes	?	Yes	Yes	Could be investigated	Morito et al., 2011
Transgenic: E $\mu$ -XBP1s	Yes	?	Yes	?	Yes	Yes	Could be investigated	Carrasco et al., 2007
Transgenic: Vk*MYC	Yes	Yes	Yes	?	Yes	Yes	Demonstrated	Chesi et al., 2008

?, no related information shown in the cited paper.

(Vanderkerken et al., 2003). The clinical characteristics of the 5T33MM and 5T2MM models – including the preferential localization of MM cells in the BM, the presence of serum M-component and increased angiogenesis in the BM – are similar to the features of human MM. In addition, both the 5T33MM and 5T2MM models were shown to have hypoxic niches in the BM (Hu et al., 2010; Asosingh et al., 2000; Azab et al., 2012). The development and use of the 5TMM models have already been well reviewed elsewhere (Asosingh et al., 2000; Vanderkerken et al., 2003).

The mouse 5T33MMvt and 5TGM1 lines are two subclonal cell lines that were established from 5T33MMvv cells, by long-term culture of the cells in vitro, so that they grow in vitro independently of the BM stroma (Radl et al., 1988; Vanderkerken et al., 2003). By injecting 5T33MMvt or 5TGM1 cells into the lateral tail vein of young C57BL/KaLwRij mice, two orthotopic MM mouse models [5T33MMvt-vv (Asosingh et al., 2000; Van Valckenborgh et al., 2002) and 5TGM1 (Goel et al., 2006)] can be established in syngeneic recipients. Because 5TGM1 and 5T33MMvt-vv cells show a similar growth pattern in the mice, we believe that they are also appropriate for studying hypoxia in MM.

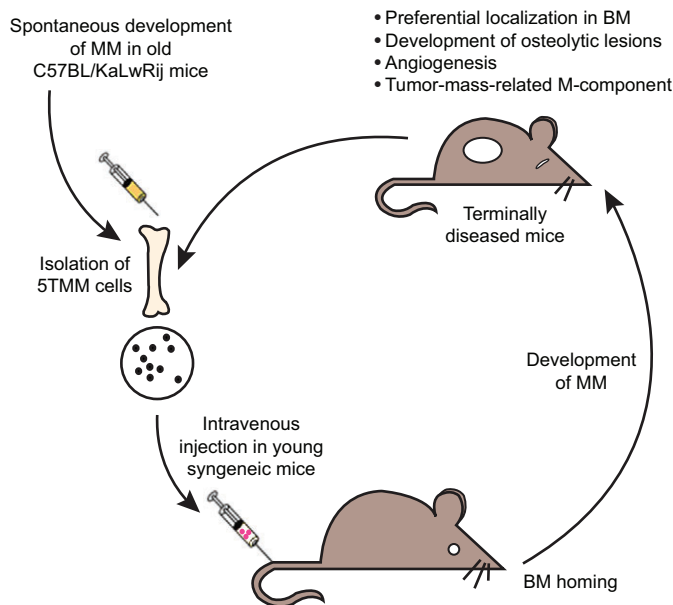
### Conventional subcutaneous SCID xenograft model

Most studies to date involving human MM cells in vivo have used human tumor xenograft models, whose development represented a major step towards more clinically relevant models of MM (Pilarski et al., 2000; Mitsiades et al., 2003). In this model, human MM cell lines are injected subcutaneously (s.c.) into severe combined immunodeficient (SCID) mice. The biggest advantage of this model

is the use of human MM cells. However, the model does not accurately simulate the in vivo behavior and drug-sensitivity patterns of human MM cells. For example, s.c. xenograft models do not reflect the systemic nature of diffuse lesions of MM. In addition, tumor cells are injected into a cutaneous microenvironment, which is radically different from the BM milieu, where MM cells preferentially home to, and which is hypoxic and promotes their proliferation, survival and resistance to therapy (Pilarski et al., 2000; Mitsiades et al., 2003). Moreover, primary myeloma cells from most patients do not grow in s.c. SCID xenograft models.

### SCID-hu model

In the SCID-hu model, SCID mice first receive implants of a human fetal bone chip, followed by injections of MM cells directly into human fetal bone grafts. Thus, the SCID-hu mouse harbors a human microenvironment in the form of human fetal bone; the human BM milieu allows the expansion and development of measurable manifestations of MM in SCID mice, including bone lesions and angiogenesis. Importantly, the MM cells grow in a (human) BM environment and, if the hypoxic nature of this MM-invaded BM is confirmed, this model can be used to investigate questions related to hypoxia in MM. However, in contrast to the rapid growth of human myeloma cell lines in SCID mice, the success of using primary MM cells has been limited in SCID-hu mice because of the following: (1) a limited number of MM cells can be harvested from an individual patient (Urashima et al., 1997; Yaccoby et al., 1998; Tassone et al., 2005); (2) the ex vivo primary MM cells cannot migrate to the mouse BM (Urashima et al., 1997; Yaccoby



**Fig. 2. 5TMM series: the natural model of MM.** MM spontaneously develops in elderly mice of the C57BL/KaLwRij strain. BM cells are isolated from diseased mice by flushing out the content of the long bones, purified by Lympholyte M gradient centrifugation and injected into the lateral tail vein of young syngeneic recipients to create 5TMM mouse models of MM. Tumor progression is monitored by serum paraprotein quantification using electrophoresis (Asosingh et al., 2000; Vanderkerken et al., 2003).

et al., 1998; Pilarski et al., 2000); (3) human fetal bone samples from donors of different gestational ages are heterogeneous in nature and quality; and (4) ethical concerns limit the use of this model in some countries.

### SCID-rabbit model

To break through the ethical concerns regarding the use of human fetal bones in the SCID-hu model, Yata and Yaccoby developed a novel system that uses rabbit bones implanted s.c. in unconditioned SCID mice, and demonstrated the ability of the rabbit BM microenvironment to attract and support sustained growth of primary CD138-selected myeloma PCs from patients (Yata and Yaccoby, 2004). Similar to the SCID-hu model, MM cells from all patients with medullary MM grew restrictively in the rabbit bone and, through interaction with the rabbit BM microenvironment, produced typical disease manifestations, such as induction of osteolytic bone lesions and angiogenesis. Given that the MM cells grow in a BM environment, this model can be used to investigate hypoxia-related questions of the MM disease, provided the hypoxic nature of the MM-invaded BM is confirmed.

### SCID-synth-hu model

To further improve the limitations of the SCID-hu model, Calimeri et al. recently reported a novel in vivo MM model whereby human bone chips were replaced with a three-dimensional (3D) bone-like poly- $\epsilon$ -caprolactone polymeric scaffold (PLCS). The authors successfully engrafted human primary explanted MM cells on the internal surface of the 3D scaffold coated with mouse or human BM stromal cells (Calimeri et al., 2011). By using these polymeric

3D scaffolds, the authors overcame the limitations of using human fetal bone chips; accordingly, the model is suitable for large-scale in vivo preclinical testing of anti-MM agents. To our knowledge, there are no available data on the hypoxic nature of these scaffolds.

### Orthotopic SCID model

By using whole-body real-time fluorescence imaging, Mitsiades et al. developed an in vivo model of MM based on tail vein intravenous (i.v.) injection of SCID mice with human MM cells stably transfected with a construct expressing green fluorescent protein (GFP) (Mitsiades et al., 2003). The anatomical distribution of GFP<sup>+</sup> lesions was consistent with the distribution of tumors in MM patients, hallmarked by the preferential development in sites of the axial skeleton (e.g. spine, skull and pelvis). Although GFP<sup>+</sup> MM cells were found to predominantly home to the bones, extraskeletal tumors (plasmacytoma of paraspinal, s.c. and visceral sites) were also observed. The model was used to investigate the migration and homing mechanisms of MM cells and to evaluate the anti-MM activity of novel agents (Mitsiades et al., 2006; Xin et al., 2006; Alsayed et al., 2007; Azab et al., 2009). This model can be used to investigate the hypoxic BM niche, and BM homing mechanisms.

### Myeloma RAG-2 knockout mice: a mouse model of MM that allows genetic manipulation of the host microenvironment

In the 5TMM mouse models, MM cells normally only grow within bone when cells are inoculated into syngeneic C57BL/KaLwRij mice (Garrett et al., 1997; Asosingh et al., 2000). However, RAG-2 [immunoglobulin (Ig) recombination enzyme] knockout C57BL/6 mice were recently shown to form 5T2MM myeloma disease in the BM. Use of this mouse strain is advantageous because it also enables easy genetic manipulation of the microenvironment. Fowler et al. developed this model with mice lacking RAG-2, enabling molecular studies of the host contribution to MM progression to be conducted in vivo (Fowler et al., 2009). Importantly, the RAG-2-deficient mice can be easily bred with genetically modified mice to generate double-knockout mice, allowing manipulation of the host microenvironment at a molecular level. Because these models are similar to the 5TMM models, and involve the growth of MM in the BM, they are suitable for hypoxia studies.

### Transgenic models

#### Modeling the human MM chromosomal translocation t(14;16)(q32;q23)

The *Maf* proto-oncogene was originally identified in the genome of the avian musculoaponeurotic fibrosarcoma virus AS42. Overexpression of *MAF* was reported in MM cells that carry the translocation t(14;16)(q32;q23), in which the immunoglobulin heavy chain (*IGH*) locus is fused with the *MAF* gene locus (Chesi et al., 1998; Hurt et al., 2004).

Although *MAF* is thought to function as an oncogene in human MM, it is still not clear how *MAF* contributes to the disease. Morito et al. showed that aged transgenic mice expressing *Maf* specifically in the B-cell compartment (using the *Igh* promoter and *E $\mu$*  enhancer) develop B-cell lymphomas with some clinical features resembling those of MM, such as PC expansion and hyperglobulinemia (Morito et al., 2011). These findings provide direct evidence that *MAF* can contribute to oncogenesis of MM.

Given that the MM cells grow in a BM environment, this model can be used to investigate hypoxia in MM.

### Modeling enforced XBP-1s expression ( $E\mu$ -XBP1s)

XBP-1 is a basic-region leucine zipper (bZIP) transcription factor of the CREB-ATF family, and a major regulator of the unfolded protein response (UPR) and PC differentiation (Iwakoshi et al., 2003; Lee et al., 2003). Although abundant expression of XBP-1 has been detected in human MM cells (Davies et al., 2003; Munshi et al., 2004), the pathophysiological relevance of the spliced isoform, XBP-1s, in MM has not been defined. Carrasco et al. developed transgenic mice with B-cell-specific ( $E\mu$ -driven) expression of XBP-1s (Carrasco et al., 2007), which resulted in the development of MGUS or MM. These mice can also be used for studying the role of hypoxia in MM.

### Vk\*MYC transgenic mice

The *MYC* oncogene has a well-known role in many tumor types. In MM, it is believed to be dysregulated by complex translocations that occur during the late stages of MM progression, not involving B-cell-specific DNA modification (Shou et al., 2000; Avet-Loiseau et al., 2007). However, earlier transgenic mouse models of forced *MYC* expression were mediated by regulatory elements of the *Ig* locus, whose activity initiates in pre-germinal center (pre-GC) B cells (Kotani et al., 2007; Park et al., 2005; Ramiro et al., 2004). These models invariably produce pre-GC lymphomas or PC neoplasms, and show little evidence of *Ig* somatic hypermutation (SHM); notably, SHM and class switch recombination of B-cell *Ig* genes only occur in activated B cells in the GC. To map *MYC* activation specifically to GC or post-GC B cells, Chesi et al. generated Vk\*MYC transgenic mice in which the activation of *MYC* is under the control of the *Ig* $\kappa$  light chain gene regulatory elements (Chesi et al., 2008; Kuehl, 2008). This system successfully modeled *MYC*-driven GC-derived plasma cell proliferation in mice. The Vk\*MYC transgenic mice universally developed post-GC PC tumors that were highly homologous to those observed in human MM. This model has been used to evaluate the drug response of the hypoxia-activated prodrug TH-302, and confirmed previous results by Hu et al. in the 5T33MM model (Chesi et al., 2012; Hu et al., 2010).

### Perspective

Despite many advances in the treatment of MM, it remains an incurable and fatal malignancy. MM is characterized by the clonal expansion of malignant PCs within the BM; signals from this microenvironment play a crucial role in maintaining MM cell growth and survival. However, our knowledge of the disease is still in its infancy and many details are unknown. As we have discussed, several recent studies have shed new light on the role of hypoxia in MM pathology, and have demonstrated that hypoxia is a promising target for MM treatment. The known importance of hypoxia in solid tumors, and the experience gained in treating solid tumors by targeting hypoxia, should boost our understanding of the role of hypoxia in MM and facilitate the development of more efficient treatment options that target the hypoxic BM niche. The in-depth investigation of the hypoxic niche in MM patients, and in existing and new animal models, will contribute to the discovery of new targets and to the acceleration of bench-to-bedside translation of new anti-MM therapies.

### COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

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