

## Review Article

# Progress in myeloma stem cells

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Received July 15, 2011; accepted July 31, 2011; Epub September 8, 2011; published September 30, 2011

**Abstract:** Multiple myeloma (MM) is the second most common hematologic malignancy in the United States and affects about 4 in 100,000 Americans. Even though much progress has been made in MM therapy, MM remains an incurable disease for the vast majority of patients. The existence of MM stem cell is considered one of the major causes of MM drug-resistance, leading to relapse. This highlights the importance and urgency of developing approaches to target MM stem cells. However, very little is known about the molecular characteristics of the MM stem cells, which makes it difficult to target MM stem cells therapeutically. Evidence of the existence of a myeloma stem cell has been provided by Matsui et al. showing that the CD138- and CD20+ fraction, which is a minor population of the MM cells, has a greater clonogenic potential and has the phenotype of a memory B-cell (CD19+, CD27+). In this review, we report recent progress of cell surface markers in cancer stem cells, especially in myeloma and the molecular mechanisms related to drug resistance and myeloma disease progression.

**Keywords:** Cancer stem cell, multiple myeloma, drug resistance, and cell signaling

### Introduction

Multiple myeloma (MM) is a hematologic cancer subtype characterized by uncontrolled proliferation of plasma cells in the bone marrow. The exact cause of MM is unknown and MM patients generally have no symptoms until the disease is already in an advanced state. In 2010, the NCI estimates that there were 20,180 new cases of MM and 10,650 deaths directly attributed to this cancer. Research into the molecular, cytogenetic, gene expression, and signaling pathway signature of MM (as reviewed and discussed in [1-4]) has significantly improved our understanding of how MM develops and progresses, and has led to advances in MM therapies and increased median survival rates. Unfortunately, this disease eventually relapses and then becomes incurable. One theory, based on the cancer stem cell (CSC) model, is that current MM treatments kill the bulk of tumor cells but not the self-renewing MM cancer stem cells (MMSCs) subpopulation. The latter type of cells is believed to be the most malignant and drug resistant but also a rare subpopulation of MM cells with the ability for self-renewal, division, and maintenance of its undif-

ferentiated state not unlike normal hematopoietic stem cells (HSCs). Although the stem cell phenotype of acute myeloid leukemia has long been identified and is generally accepted, the definitive MMSC phenotype / markers are still being deliberated in the MM research community and there are currently several viewpoints on MM cells with stemness characteristics. Targeting of MMSCs is clinically relevant since insight into the characteristics of this subpopulation, if it exists, could result in novel therapeutics that can make MM a more manageable and even curable disease.

### Normal and cancer stem cells in the hematopoietic system

The existence of HSCs is an accepted concept along with their important use clinically (as reviewed in [5, 6]). HSCs are typically isolated based on their surface markers as first shown by the Weissman group for both mouse and human stem cells [7, 8], and it has now been 10 years since it was demonstrated that a single bone marrow-derived cell can recapitulate blood cell lineages in irradiated mice [9]. Gene expression profiling (GEP) of highly purified

HSCs versus non-self-renewing multipotent hematopoietic progenitors helped identify the SLAM family of cell surface markers, and has further improved surface marker-based fluorescence activated cell sorting (FACS) to almost 100% purity with about half of the purified fraction possessing full HSC activity [10]. The relatively successful isolation of semi-pure HSC has been translated into clinical use (as reviewed in [5, 6]). HSCs are critical in stem cell transplantation (SCT) after myeloablative therapy to treat various diseases including leukemia and multiple myeloma [11]. With the exception of myeloma, SCT are mainly performed upon relapse, which could reflect the resistance of CSCs to traditional cancer therapies (the relevance of CSCs to BMT is discussed in [12]). Although the idea of the clonal origin of malignancies and the still utilized clonogenesis assay for CSCs have been around for more than 40 years now [13-15], definitive evidence for hematopoietic CSCs and its hierarchical organization was only first presented by Bonnet and Dick in 1997 based on their seminal observation that only the CD34<sup>+</sup>/CD38<sup>-</sup> leukemia cell subpopulation is able to induce leukemia in a SCID mouse model [16]. The unique MMSC signature, unfortunately, is still unresolved. While the markers for different CSC types differ from one another, there is congruence in their functional properties including ability for self-renewal, tumorigenesis, and drug-resistance. Thus, these stemness characteristics are the basis for trying to functionally identify the definitive MMSC signature.

### Multiple myeloma stem cells

HSCs are different from CSCs in that the former's activity and function are tightly regulated whereas they are dysregulated in CSCs, which eventually lead to cancer cell overpopulation (where the CSC remains a minor fraction). In MM, this minor population has functionally been shown to have increased potential of self-renewal. The cancer stem cell model is based on the concept that most cancers just like the normal hematopoiesis, are hierarchically organized [17]. This implies that CSCs give not only to daughter cancer cells that are more differentiated, but also to another CSC (asymmetrical division). Based on this model, MMSCs then should possess a surface marker signature more similar to plasma cell precursors. Indeed drug-resistant clonotypic cells from myeloma

patients with stemness characteristics have been reported to have memory B cell-like surface marker profile [18-21] although MM cells with stemness characteristics not possessing this signature have also been reported [22, 23].

HSC and CSC research share a common problem that these rare cells being very difficult to identify, isolate, and characterize. The possible existence of MMSCs was first postulated based on experiments using MM cell lines and patient MM samples indicating that there is a small population of MM cells with the ability for unlimited self-renewal [24, 25]. Second, the capacity for self-renewal of primary MM samples *in vitro*, using the sphere-forming assay [26], as well as *in vivo*, by serial transplantability in NOD/SCID mice [27] was also observed. Finally, the fact that most MM patients after traditional treatment ultimately relapse also points to the existence of MMSC.

The identification of SC markers in combination with FACS has exponentially increased the efficiency of HSC and CSC identification and isolation. This has led to progress in trying to identify the unique MMSC surface marker signature.

1) The SCID-hu *in vivo* mouse model and FACS were used by Yaccoby et al. to show that only primary CD38<sup>+</sup>/CD45<sup>-</sup> plasma cells proliferate successfully within the engrafted fetal bone microenvironment although it did not invade the murine host bone [22] and CD38<sup>+</sup> is not a plasma cell precursor marker *but* is a plasma cell marker. However, the purity of the population is never 100% and it is possible that the disease was propagated by the few CD 138<sup>+</sup> cells.

2) Drug resistant MM cells in a novel 3D model were CD20<sup>+</sup>, although expression of CD20 is heterogeneous in bulk myeloma cells and is only detected in a minority of patients [28].

3) Cells with the CD138<sup>+</sup>/CD19<sup>+</sup>/CD20<sup>+</sup>/CD27<sup>+</sup> phenotype which is memory B cell-like, are serially transplantable. This is consistent with the theory that CSC can arise from hierarchically more primitive than malignant plasma cells [18, 19, 29].

4) The CD138<sup>+</sup> MM phenotype has also been associated with increased apoptosis of these cells [30]. It is now generally accepted that

**TABLE 1.** Cancer stem cell markers

Cancer	CD19	CD20	CD24	CD27	CD34	CD38	CD44	CD133	CD138	CD166	ALDH1	ESA	other
breast			- / low				+	+			+	+	B1, Lin-
brain								+					
colon							+			+		+	
head and neck							+						
leukemia (ALL)					+	-							t(12;21), Lin-
leukemia (AML)					+	-							t(9;22), Lin-
lung								+			+		
melanoma		+						+					CD271, Hoescht 33342 SP
pancreatic			+				+					+	
prostate							+	+			+		B1
ovarian													Hoescht 33342 SP
multiple myeloma	+	+		+					-		+		Hoescht 33342 SP

Table modified from [37-39]. B1 = integrin B1 chain; ALDH = aldehyde dehydrogenase; ESA = epidermal surface antigen

apoptotic myeloma cells lose CD138 marker on its membrane.

5) Yaccoby showed that a co-culture of differentiated MM cells with OCs reprogrammed the differentiated MM cells to immature CD138<sup>low</sup> MM cells that were drug resistant [31].

6) Pilarski et al. showed CD34<sup>+</sup>/CD45<sup>low</sup> clonotypic myeloma cells xenograft in NOD/SCID mice [32]. A subpopulation of cycling CD34<sup>+</sup>/CD138<sup>+</sup>/B7<sup>-</sup>/H1<sup>+</sup> mature plasma cells from primary MM patients was shown to be malignant and could be a possible source of mature MM plasma cell de-differentiation to MMSC [33]. This intriguing possibility was also raised by results recently published by the Weinberg group showing that neoplastic mammary epithelial cells spontaneously convert to a stem-cell like state [34].

7) Aside from surface markers, FACS has also been used to sort MMSC subpopulation based on functional stem cell markers including ALDH expression [35] and efflux pump ABCG2 activa-

tion in a side population (SP) by Hoechst 33342 staining [36].

8) Jakubikova et al. recently showed that various MM cell lines contain SPs that are inhibited by lenalidomide and that this SP has no correlation with CD138<sup>-</sup> [23] but Matsui et al. , in an earlier publication, showed that ALDH<sup>+</sup> and SP are enriched for CD138<sup>-</sup> MMSC [18].

MMSC stem cell markers agreeable with the hierarchal model of CSC theory, along with the stem cell markers of various types of malignancies, are shown in **Table 1** [37-39]. The MM subpopulations with stemness features above were tested *in vivo* using SCID mouse models that permit proliferation initiated by clonogenic myeloma cells (except for [28]). The robust *in vivo* NOD/SCID model was also used to verify most of the cancer stem cell markers listed in **Table 1** [37]. A related model, the SCID-hu, which involves implantation of fetal bone and injection of myeloma cells in this “pseudo-synergistic” humanized environment, was used by Yaccoby et al. to identify the clonogenic potential of the

CD38<sup>+</sup>/CD45<sup>-</sup> population [22]. One criticism against the SCID models is that the clonogenic growth of the transplantable subpopulations could be dependent on the recipient's weakened immune system. A recent study seems to support this hypothesis. The use of a more severely immunocompromised model, the IL2 $\gamma$  null NOD/SCID mice, led to at least a 250-fold increased tumor formation *in vivo* compared to NOD/SCID mice [40, 41]. The MM-specific 5T33 or 5T2 murine mouse model will be useful in confirming the validity of currently published MMSC markers in identifying MMSC since the clonogenicity of the injected murine myeloma cells will be dependent on, and synergistic to, the recipient bone marrow microenvironment (as further discussed in [42-44]).

There is currently a major controversy regarding the MMSC signature, although this issue is not unique to myeloma [21]. Methodological differences in stem cell purification and source undoubtedly perturb the primary stem cell niche [45] to different extents depending on the protocol employed and could contribute to the identification of various MM subpopulations with stemness characteristics. To definitively confirm self-renewal of the proposed MMSC populations (compared to differentiated but highly proliferative cells) above, *in vitro* assays need to be done at the single cell level together with the appropriate *in vivo* model.

## MMSC in MM treatment and relapse

Intensive research on the molecular mechanisms of MM disease and progression has led the discovery of a new generation of therapeutics against MM. The thalidomide derivative, lenalidomide, has recently been shown to be effective against relapsed and refractory MM and has been experimentally shown to target the MM SP [23]. The 26S proteasome inhibitor, bortezomib, significantly delays MM progression compared to dexamethasone [46] by inducing apoptosis and inactivation of upregulated NF $\kappa$ B [47-49] although MM cells with constitutively activated NF $\kappa$ B expression are resistant to bortezomib [50]. Synergistic effects have been observed when these drugs are used in combinatorial therapy [51, 52]. Even with more available treatment options and a better understanding of the molecular underpinnings of MM, MM remains a largely incurable disease. In general, current systemic cancer therapies fail to

cure advanced tumors, and MM is no exception. Based on CSC theory, MM relapse can be explained by the persistence of cancer stem cells after chemotherapy.

## Targeting MMSC

Beside the distinctive properties of self-renewal and proliferation, cancer stem cells are thought to be much more resistant to chemo- and radiotherapy and are a logical cause of drug resistance [17,53,54]. MM is no exception as most anti-MM drugs prolong survival but do not cure the disease. The SCID-hu model is used to test anti-MM drugs *in vivo* [55-57] but not specifically against the MMSC subpopulation. The CD138<sup>+</sup>/CD19<sup>+</sup>/CD20<sup>+</sup>/CD27<sup>+</sup> MM stem cells have been shown to be resistant to dexamethasone, lenalidomide, cyclophosphamide and bortezomib [18]. However, in other studies, the MM SP stem cells were shown to be susceptible to lenalidomide treatment [23]. These results indicate that while lenalidomide can kill potential MMSCs, it may still be ineffective against other possible MMSC subpopulations. One possible solution is to target the self-renewal pathway specific for CSCs. These include the Hedgehog (HH), Wnt, and Notch pathways which are typically active in cancer stem cells [29, 17, 58-63]. One study in particular showed that the HH pathway through SMO upregulation allows for clonal expansion of MM CD138<sup>+</sup> cells *in vitro*, while inhibition of CD138<sup>+</sup> proliferation was observed by using the HH pathway inhibitor cyclopamine [29]. Numerous CSC-targeted therapeutics, specifically inhibiting the HH / Wnt / Notch self-renewal pathways, are now currently in clinical trials including two specifically for MM patients after autologous stem cell transplantation (**Table 2**).

A major reason of failure in cancer treatment is the existence of a drug resistant sub-clone, which may represent cancer stem cells at diagnosis and may expand during treatment. A comprehensive analysis of the genetic and molecular mechanisms of drug resistances in cancers remains elusive. We were the first to describe that the presence of chromosomal abnormalities detected by conventional metaphase cytogenetic analysis, as the most important predictor for outcome in myeloma [64]. In particular, complete or partial deletion of chromosome 13, present in 17% of newly diagnosed myeloma patients, was associated with poor prognosis

## Myeloma stem cells

**Table 2.** Potential cancer stem cell self-renewal pathway inhibitors

Drug	Company / Institute	Pathway	Indication	Combination drug	Phase
LGK974	Novartis Pharmaceuticals	Wnt	melanoma, breast neoplasms, lobular carcinoma		I
PRI-724	Prism Biolab Corp	Wnt	advanced solid tumors		I
Resvaratrol	Univ of California, Irvine	Wnt	colon cancer		I / II
Diclofenac + Vitamin D3	Maastricht Univ Medical Center	Wnt + HH	basal cell carcinoma		III
R04929097	Cancer Institute of New Jersey / NCI	Notch	multiple myeloma and plasma cell neoplasm after autologous stem cell transplant	melphalan	II
R04929097	M.D. Anderson Cancer Center / NCI	Notch	lung cancer after front-line chemotherapy		II
R04929097	Albert Einstein College of Medicine of Yeshiva Univ / NCI	Notch	cutaneous melanoma		II
R04929097	Children's Oncology Group / NCI	Notch	brain and central nervous system tumors leukemia, lymphoma, unspecified childhood solid tumor	dexamethasone for all indications	I / II
PF-04449913	Pfizer	HH	solid tumors, unspecified hematological malignancies		I
PF-04449913	Pfizer	HH	chronic myeloid leukemia	dasatinib, bosutinib	I
LEQ506	Novartis Pharmaceuticals	HH	advanced solid tumors, recurrent or refractory medulloblastoma		I
IPI-926	Infinity Pharmaceuticals	HH	metastatic pancreatic cancer, recurrent head and neck cancer	gemcitabine, cetuximab	I / II
GDC-0449	Roche Pharma AG Genentech	HH	conventional chondrosarcoma various indications including MM**	various interventions**	II I / II

\*\*From clinicaltrials.gov July 2011: <http://clinicaltrials.gov/ct2/results?term=gdc-0449>

[65]. This was surprising since such a deletion by fluorescence in-situ hybridization (FISH) analysis is associated with a good prognosis in chronic lymphocytic leukemia (CLL) [66]. Using FISH analysis, many of the partners involved in translocations with 14q32 (heavy chain gene locus) have been identified, some of which could not be detected with conventional cytogenetics, because of their telomeric location [67]. These include t(4;14) involving FGFR3, t(14;16), involving c-MAF and t(14;20), involving MAF-B. We now know that these translocations have a poor prognosis and are very often associated with a deletion of chromosome 13. Therefore, the poor prognosis of chromosome 13 by conventional cytogenetics is most likely not directly related to loss of cytogenetic material of this chromosome, but to the frequent association with these poor-prognosis translocations defined by FISH [68]. These translocations are much more frequently seen in hypodiploid myelomas, which are known to have a poor prognosis [69]. Large studies using FISH analysis also identified that a deletion of 17p13 (p53 gene locus) was a poor prognostic indicator. In con-

trast, hyperdiploidy or translocations involving Cyclin D1, t(11;14) or Cyclin D3, t(6;14) have a good prognosis. Cyclin D abnormalities are an early event in the development of myeloma [70]. Although cytogenetic abnormalities are more frequently (almost in 100% of patients) found with FISH analysis, the predictive value of metaphase abnormalities, found in one third of patients has proven to be superior to that of FISH [71], because they represent the best surrogate marker for bone marrow stroma dependency of the myeloma cells. Typically, if myeloma cells are removed from their micro-environmental support system and transferred to a hostile environment in the test tube, they rapidly apoptose. The mitoses observed in such patients by conventional cytogenetics are derived from the remaining normal hematopoietic cells. If on the other hand, myeloma cells have become stroma-independent, which is a feature of advanced and aggressive myeloma, they will not undergo apoptosis after removal from the bone marrow stroma, and will be able to survive and grow in the test tube and thereby will give rise to abnormal metaphase cytogenetics [72].

Global gene expression profiling (GEP) has emerged as a powerful tool for classifying disease subtypes, for developing robust prognostic models and identifying new drug-resistance targets in cancers [73-76]. Using GEP analysis, we have established transcriptomes by comparing multiple myeloma (MM), monoclonal gammopathy of undetermined significance (MGUS), and normal plasma cells [77, 78]. We have elucidated the role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions [79, 80]; and identified molecular and genetic myeloma subgroups [3, 81]. We have also defined a 70-high-risk gene model through the GEP analysis of more than 550 newly diagnosed MM patients [4]. Through GEP analysis, our lab determined that *CKS1B* gene, which is in the 1q21 chromosome amplification hotspot in MM and is overexpressed in relapsed patients, is associated with increased drug resistance and is a viable anti-myeloma target [82-84]. Using fluorescence in situ hybridization (FISH) we investigated amplification of chromosome band 1q21 (Amp1q21) in 479 untreated MM patients and 22 MM cell lines. The frequency of Amp1q21 was 43% in newly diagnosed MM, but was present in 21 of 22 (95%) in MM cell lines. We also correlated microarray data with FISH results in plasma cells from 250 patients. This analysis revealed that the percentage of cells with amplification of the *CKS1B* locus was a significant predictor of *CKS1B* gene expression level ( $P < 0.0001$ ). Newly diagnosed MM patients with Amp1q21 had an inferior 5-year overall survival compared with those lacking Amp1q21 ( $P < 0.001$ ). Also, patients, who had the highest expression of *CKS1B* (top 13% of 351 patients) at baseline and at relapse, had the worst 5-year overall survival when receiving salvage therapies ( $P = 0.0025$ ). We further demonstrated that *CKS1B* plays a crucial role in MM cell growth and survival and for the first time provides direct evidence for the crucial role of *CKS1B* in myeloma multidrug-resistance [84]. We identified STAT3 and MEK/ERK/BCL2 as *CKS1B*-downstream signaling pathways; and thereby provided targets for the development of new therapeutic approaches for *CKS1B* over-expressing myeloma and other malignancies [84].

GEP was also utilized in MMSC CD138<sup>-</sup> and CD138<sup>+</sup> cells isolated from ARP.1, CAG, H929, JJN3, KMS28PE, OCI-MY5, OPM2, U266 and XG1 cell lines and we identified 645 genes that

were significantly differentially expressed in CD138<sup>-</sup> cells compared to CD138<sup>+</sup> cells (SAM,  $FDR < 1\%$ ), with 598 up-regulated and 47 down-regulated. Our preliminary data based on analysis of 9 MM cell lines show that RAR $\alpha$  is the top one up-regulated gene in CD138<sup>-</sup> MM stem cells. RAR $\alpha$  has two major isoforms, RAR $\alpha$ 1 and RAR $\alpha$ 2. Real-time PCR detected significantly higher expression of RAR $\alpha$ 2 but not RAR $\alpha$ 1 in CD138<sup>-</sup> MM stem cells compared to CD138<sup>+</sup> tumor cells. While RAR $\alpha$ 1 was ubiquitously expressed in MM cells, RAR $\alpha$ 2 was present in only 30% of newly diagnosed patients [85]. Patients with RAR $\alpha$ 2 expression had a significantly shorter overall-survival, also on multivariate analysis. RAR $\alpha$ 2-knockdown in RAR $\alpha$ 2<sup>+</sup> MM cells induced cell growth arrest and apoptosis [85]. Wnt signaling is activated in MM stem cells and forced-RAR $\alpha$ 2-expression in MM cells induced Wnt activation. These results suggest that RAR $\alpha$ 2-mediated Wnt signal activation may be critical for MM stem cell survival and MM progression. Both RAR $\alpha$ 1 and RAR $\alpha$ 2 are specific receptors for all-trans retinoic acid (ATRA). Interestingly, we found that ATRA selectively killed MM stem cells while sparing CD138<sup>+</sup> tumor cells. Forced expression of RAR $\alpha$ 2 in RAR $\alpha$ 2-deficient MM tumor cells restored sensitivity to ATRA. Finally, ATRA was shown to down-regulate Wnt signaling ( $\beta$  catenin and other downstream targets).

We also compared GEP samples at baseline, in remission, and at relapse from patients enrolled on our 25009 protocol, and defined 56 genes associated with drug resistance (persistence after transplantation) or rapid disease relapse ( $< 2$  years after the second transplant). The chromosomal instability signatures (CINs) were identified as the most differentially expressed genes, with low expression at diagnosis and high expression after chemotherapy (at a stage with minimal residual disease) and at relapse. Our functional assays indicate that increased CIN signature promotes MMSC self-renewal and induces drug resistance by up-regulating ABCG2 efflux and the Wnt pathway (submitted manuscript). We are currently working on verifying these novel MMSC-specific targets with the goal of using these findings in future MM therapies.

However, even if a potential drug candidate is found that specifically targets the self-renewal pathways, there must also be a more selective targeting of MMSC compared with normal HSC.

Better targeted combinatorial therapy using the self-renewal pathway inhibitor plus either traditional chemotherapeutic drugs (as in **Table 2**) or a selective inhibitor of MMSCs and not HSCs needs to be developed. For example, a potential target, cell surface receptor CS1, is highly expressed in the bulk of the myeloma cells but not HSC, has been targeted successfully with a monoclonal antibody that blocks CS1 activity and inhibits MM cell bone adhesion [86, 87]. Antibodies raised against CD44, an acute myeloid leukemia SC marker, successfully eliminated CSCs *in vivo* in a NOD-SCID mouse model [88]. Similarly, in MM, CD20 antibody was demonstrated to inhibit CD138<sup>+</sup> clonogenic recovery in a complement-dependent manner [18] although CD20 is only expressed in a minority of MM patients and response to CD20 antibody treatment is variable as tabulated by Kapoor et al. [89].

## Future directions

What is a cancer stem cell? Ideally the answer should only depend on the type of cancer, but currently in MM the CSC phenotype is still poorly defined as is the concept of CSC itself. Even if the MMSC signature is found, there are still many questions to answer: Can MMSCs be expanded in *in vitro* cultures? How do MMSCs interact with the bulk tumor? What is its niche? Are the MMSC always the same cells or is there plasticity as has been shown in HSC?

The physical isolation of the MMSC intrinsically disturbs the CSC niche and could contribute to the differences in phenotype being observed but it is still of vital importance to identify this (these?) subpopulation(s) if a cure for MM is to ever be found. Single cell *in vitro* assays can be used to compare the absolute clonogenicity of the various potential MMSC populations. A distinct advantage of studying MM is that the HSC system is well established and the blood cancer leukemia stem cell system serves as a guide for elucidating the MMSC system. If a definitive MMSC signature / marker attains universal acceptance, microarray profiling could be used to associate this signature / marker with a prognosis for MM patient survival. The Morrison group used GEP to find the leukemia SC marker SLAM [10]. Similarly, our group has performed GEP between new and relapsed patients, and between CD138<sup>+</sup> and CD138<sup>+</sup> MM cell lines and have found promising targets that seem to be

involved in drug resistance and self-renewal [84, 87, 90]. Targeting of MMSC will also have a different therapeutic profile than traditional drugs, and attacking the bulk tumor, the stem cell niche, and the microenvironment needs to be considered especially if the more differentiated bulk tumor might have the capacity to revert to the stem cell-like phenotype [31, 34].

## Acknowledgements

This work was supported by National Cancer Institute R01CA115399 (GT), R01CA152105 (FZ), R21CA143887 (FZ), leukemia lymphoma society (FZ), Multiple Myeloma Research Foundation (FZ 2008 & 2010), and institutional start-up funds from the School of Medicine and Huntsman Cancer institute of the University of Utah (FZ).

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