Chapter

3D Models of Surrogate Multiple Myeloma Bone Marrow Microenvironments: Insights on Disease Pathophysiology and Patient-Specific Response to Drugs

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Abstract

Multiple Myeloma (MM) develops almost exclusively within the Bone Marrow (BM), highlighting the critical role of the microenvironment in conditioning disease progression and resistance to drugs. Indeed, while the therapeutic armamentarium for MM has significantly improved over the past 20 years, the disease remains ultimately incurable. This failure may depend on the high phenotypic and genetic heterogeneity of MM, but also on the paucity and inadequacy of two-dimensional (2D) conventional preclinical models in reproducing MM within the BM. In the present paper, we provide a brief updated overview on MM BM microenvironment. We then discuss newly developed preclinical models mimicking MM/microenvironment interactions, including three-dimensional (3D), gel-based, *in vitro* models and a novel *ex vivo* system of isolated tumor and stromal cells cultured in bioreactor. Potential applications of each model, relative to investigation of MM pathogenic mechanisms and prediction of the best drug/combination for each individual patient will be also evaluated.

Keywords: multiple myeloma, tumor microenvironment, 2D/3D culture models, 3D culture in bioreactor, drug testing, personalized therapy

1. Introduction

Multiple Myeloma (MM) is a B-cell tumor characterized by clonal proliferation of malignant plasma cells (PC) inside the bone marrow (BM), production of a monoclonal paraprotein and associated clinical features, including hypercalcemia, renal failure, anemia and lytic bone lesions (CRAB features) [1, 2].

MM is the second most common hematological malignancy and is responsible for approximately 20% of deaths from hematological tumors. Despite significant advances in therapy over the past two decades, the disease remains incurable, and more than 90% of MM patients eventually become refractory to therapy and relapse [1, 2].

MM develops along an evolutionary process, leading a normal PC to the pre-malignant state of monoclonal gammopathy of uncertain significance (MGUS),

an intermediate asymptomatic but more advanced pre-malignant state referred to as smoldering MM (SMM) and finally to symptomatic MM [3–6]. This process is driven by the accumulation of cytogenetic modifications in PC. Indeed, while MM is still considered a single disease entity, it should be viewed as a collection of several different cytogenetically distinct PC tumors [7]. Cytogenetic abnormalities encompass translocations involving the immunoglobulin heavy chain (IgH) gene locus on chromosome 14q32 and hyperdiploidy (particularly trisomies), as initiating events [8]. IgH translocations include t(4;14), t(6;14), t(11;14), t(14;16) and t(14;20) translocations, which place the oncogenes Multiple Myeloma SET domain (MMSET)/ fibroblast growth factor receptor 3 (FGFR3), cyclin D3 (CCND3), CCND1, MAF, and MAFB, respectively, under the control of the strong enhancers of the Ig loci. This in turn leads to over-expression of cyclin D protein family members, ultimately driving G1/S checkpoint dysregulation [9, 10]. Hyperdiploidy, which is associated with the gain of the odd numbered chromosomes, including chromosome 3, 5, 7, 9, 11, 15, 19 and 21, also affects this checkpoint, implicating cyclin D dysregulation as an early and unifying oncogenic event in MM [9]. Subsequent studies demonstrated that other cytogenetic changes termed secondary cytogenetic abnormalities, including gain(1q), del(1p), del(17p), del(13), RAS mutations and secondary translocations involving MYC, arise along the disease course of MM, exacerbating the cell cycle dysregulation and driving further proliferation and disease progression [10, 11]. Patients carrying del(17p), t(4;14), t(14;16), t(14;20), gain(1q), or p53 mutation, particularly when in combination (double-hit and triple-hit myeloma), are considered affected by high-risk MM [11], and represent an area of unmet clinical need [8].

In addition to genetic abnormalities, a characteristic feature of myeloma cells is the requirement for an intimate relationship with the BM microenvironment, where plasma cells are nurtured in specialized niches that maintain their long-term survival. Indeed, BM components deeply influence many steps of tumor progression, such as MM proliferation and invasion, angiogenesis and drug resistance [12, 13].

The BM, where MM cells specifically home mainly through the CXCR4/CXCL12-SDF1α axis [14], provides a highly specialized microenvironment, which optimally "soils" neoplastic PC, and, in turn, is shaped by the interactions with the tumor [15, 16]. The BM microenvironment comprises two major compartments, *i.e.*, the cellular and the non-cellular compartment. The latter includes the extracellular matrix (ECM), consisting of collagen I to XI, fibronectin, glycoproteins, matrix proteoglycans and glycosaminoglycans, as well as the liquid milieu (cytokines, chemokines and growth factors). The cellular compartment consists of a series of components, including BM stromal cells (BMSC), hematopoietic cells, osteoclasts, osteoblasts, endothelial cells (EC), adipocytes and immune cells. Inside the BM milieu MM cells realize a complex interplay involving both cellular and ECM components through the engagement of adhesion molecules and the release of soluble factors, including cytokines, growth factors and exosomes [12]. Exosomes are extracellular membranous vesicles known to facilitate the transfer of biologically active molecules, including proteins and nucleic acids (particularly microRNAs -miRNAs), from the original producing cell to the target cell [17]. Exosomes are released by almost all cell types and, depending on their cargo, can induce target cell activation, proliferation/differentiation or death, thus playing a key role in the regulation of physiological as well as pathologic processes, including malignant transformation [17]. In MM, exosomes have been recently shown to reprogram the BM microenvironment, creating a niche for tumor PC and favoring their expansion and the onset of pharmacological resistance [18–20].

Another key feature of the BM microenvironment is hypoxia. In the BM, oxygen (O_2) tensions fluctuate throughout two specialized niches, the hypoxic endosteal niche and the oxygenated vascular niche, mapping areas with controlled, physiological O_2 gradients, instrumental to hematopoietic stem cells homeostasis [21]. BM homing is a common feature of hematological malignancies, that in proximity of hypoxic niches escape drug-inflicted apoptosis and acquire a drug-resistant phenotype. This is particularly true for MM that develops almost exclusively in the BM, where myeloma cells accumulation and the abnormal vasculature contribute to aggravate hypoxia. BM samples from MM patients as well as circulating MM cells are reported to have a hypoxic phenotype [22] and a strong stabilized expression of the hypoxia master regulator hypoxia-inducible factor (HIF)-1α protein [23]. Notably, HIF-1α suppression in myeloma cells blocks tumor growth *in vivo* and interferes negatively with angiogenesis and bone destruction [24]. In addition to conventional cell contact-dependent and -independent signaling pathways, hypoxia promotes MM survival and drug resistance through alternative mechanisms. Hypoxia is indeed a major regulator of exosomal content and angiogenesis in MM settings [25]. Moreover, hypoxia shifts the metabolic profile of MM cells toward elevated glycolysis and production of lactate, as a strategy to support energy requirement [26]. Notably, knockdown of lactate restores MM sensitivity to bortezomib, overall suggesting that targeting hypoxia and MM energy metabolism could alleviate drug resistance [26].

Overall, the cross-talk between MM cells and their BM microenvironment results in autocrine/paracrine loops of MM survival/proliferation and also promotes the "angiogenic switch", osteoclastogenesis, and defective immune functions [12, 13]. In particular, adhesion of MM cells to ECM components and to BMSC triggers classical survival signaling pathways including, but not limited to, the PI3K/AKT signaling pathway, anti-apoptotic signals and also the release of the pro-survival factor Interleukin (IL)-6 [27]. MM cells-BM interactions also play a key role in disease pathogenesis. In particular, new blood vessel formation is considered a hallmark of MM development and is supported by the histopathological evidence of increased microvessel density (MVD), surrogate parameter endowed with prognostic significance, in the BM of MM patients [28]. Angiogenesis, the sprouting of capillaries from existing blood vessels, is also suggested by the plethora of soluble angiogenic factors in the BM and in the peripheral blood (PB) samples from myeloma patients (vascular endothelial growth factor, VEGF; basic fibroblast growth factor, bFGF; angiopoietins, Angs) [29, 30], whose contribution to the process has been extensively reviewed [31]. Moreover, the finding of an elevated number of circulating endothelial precursor cells (EPC) in MM patients indicates that complementary modalities to build vessels, e.g., vasculogenesis, are engaged [31]. Finally, EC are by nature fine sensors of O_2 variations, and the hypoxic microenvironment inside the BM significantly contributes to the induction of the "angiogenic switch" and the maintenance of the pro-angiogenic profile through the transcription of HIF-1α [32].

MM plasma cells and BM stroma also contribute to the pathophysiology of MM-associated bone disease through the activation of signaling pathways regulating osteoclastogenesis, particularly the RANK/RANK-Ligand (RANK-L) and the Wnt pathway, and the release of osteoclast-activating factors, such as IL-1, IL-6, tumor necrosis factor (TNF)-α, IL-8 and Macrophage Inflammatory Protein (MIP)-1α. These factors, together with recently identified dysregulated miRNAs, determine osteoblast suppression with excessive osteoclastic resorption [33]. Finally, MM cells display a unique ability to evade immune surveillance through several mechanisms, including impairment of cytotoxic activity, induction of dendritic cell dysfunction and recruitment of regulatory cells [34].

2. 3D models of MM microenvironment for precision medicine

2.1 Therapeutic targeting of MM cells and their BM microenvironment: toward personalized therapy

Over the past 20 years, progressive understanding of the pathophysiology of MM has informed treatment paradigm and patients' outcome [35]. In particular, the introduction into the clinical practice of novel agents, such as proteasome inhibitors (PI) and immunomodulatory drugs (IMiDs), has prolonged median survival of MM patients from 3 to about 6 years, reaching approximatively 8 years in the subset of patients eligible to autologous stem cell transplantation (ASCT) [11].

The proteasome inhibitor bortezomib targets MM cells harnessing their dependency on the protein quality control pathway as a therapeutic target [36]. The ubiquitin-proteasome system represents a major mechanism for maintaining protein homeostasis, which is strictly required by normal antibody secreting PC, and particularly by MM PC [36]. Bortezomib causes an imbalance between proteasome degradative capacity and proteasome load, leading to the activation of the unfolded protein response, and ultimately to cell death *via* both intrinsic and extrinsic mechanisms of MM cell apoptosis [37]. Moreover, bortezomib affects viability of angiogenic EC [38], as well as bone turnover and osteoclast activity in the BM [39].

Given the key role of BM components in supporting MM cell proliferation, migration, survival and drug resistance, while also conferring immunosuppression, disrupting MM cells-BM interactions represents an alternative therapeutic strategy in MM. IMiDs, including thalidomide and its more potent derivatives lenalidomide and pomalidomide, have received Food and Drug Administration (FDA) approval for treatment of both newly diagnosed and relapsed/refractory MM [35]. IMiDs bind to cereblon (CRBN) and activate CRBN E3-ligase activity, causing the rapid ubiquitination and degradation of two specific B cell transcription factors, Ikaros (IKZF1) and Aiolos (IKZF3) [40, 41]. IMIDs thus exert direct cytotoxic effects on MM cells, including growth arrest, free radical-mediated DNA damage and caspase-8-mediated apoptosis; moreover, they modulate cytokine and growth factor secretion, inhibit angiogenesis, and, most importantly, upregulate T, NK, and NKT cytotoxicity, while downregulating regulatory T cells [42].

Over the disease course, however, MM cells acquire resistance to bortezomib and IMiDs through genetic and non-genetic mechanisms [36, 43]. To overcome resistance, second-generation PI (carfilzomib, ixazomib) and higher affinity CRBN E3-ligase modulators, such as iberdomide, have been developed [35, 36]. Alternative therapeutic approaches include: targeting epigenetic modifications *via* the Histone deacetylases (HDAC) inhibitors (panobinostat, ricolinostat); targeting the tumor-BM microenvironment interface *via* immune-based therapies, including monoclonal Antibodies (mAb) directed against MM surface antigens (elotuzumab and daratumumab, targeting SLAMF7 and CD38, respectively) and cellular therapies to boost MM- specific immunity, including adoptive T-cell therapy (ACT), engineered T-cell approaches and vaccines [35, 44]. Notably, progress in engineering technologies allowed for chimeric antigen receptor (CAR) T-cell approaches [45]. CAR are chimeric proteins that bring together the signaling moieties of the T cell receptor (TCR) complex and the variable domains of an Ab recognizing a tumor-associated antigen (in MM, most frequently the B cell maturation antigen –BCMA-, due to its selectivity for normal PC and MM cells) [35]. As a result, in the last decade, carfilzomib, pomalidomide, panobinostat, ixazomib, elotuzumab, daratumumab, isatuximab, and selinexor (a selective inhibitor of nuclear export of tumor suppressor proteins and growth factors) have received FDA approval for the treatment of relapsed MM, and are expected to improve outcomes further [11].

To date, therapy for an individual MM patient is selected based on clinical factors, such as age, performance status, comorbidities and eligibility for ASCT [46]. Given the high heterogeneity of the disease in terms of underlying molecular aberrations and clinical course, and also the growing armamentarium of currently available effective agents, this approach can be updated by the use of evidence-based algorithms [46], but it also needs to be implemented by incorporating prognostic and predictive biomarkers for survival and response to treatment [8]. Indeed, thanks to the progressive evolution and clinical utilization of molecular technologies, such as fluorescence in situ hybridization (FISH) and next-generation sequencing (NGS), we can foresee that in the near future the choice of therapy may include selection of targeted treatments based on the presence of specific molecular lesions, thus achieving personalized cancer care for MM patients [8]. Such treatments can be validated through randomized controlled clinical trials [8]; however, the development of reliable patient-specific pre-clinical models would also be valuable in the perspective of defining personalized, biologically based treatments for MM patients, while preventing ineffective therapy of resistant MM cells and unwanted toxicities [47].

2.2 *In vitro* models of cancer: moving from 2D to 3D

It is increasingly recognized that microenvironment plays a fundamental role in supporting tumor cell growth, survival and drug resistance; thus, experimental models of cancer should incorporate elements of the surrounding milieu to recreate and unveil the mechanisms that, at the molecular level, regulate the complex interplay between tumor cells and their embedding niche(s).

Traditional two-dimensional (2D) *in vitro* cultures, *i.e.*, static cultures of cells kept on flat, artificial surfaces, still represent the most popular models for *in vitro* studies. These culture systems have so far provided invaluable information on the basic molecular principles of cancer; it is becoming progressively clear, however, that they present severe limitations, since they fail to reproduce adequately morphology, behavior, and functions of normal and pathologic cell types and tissues [48]. It is now generally agreed that the generation of reliable and physiologically relevant *in vitro* tissue analogues, tumors included, should rely upon reproducing (or preserving) the specific characteristics of the native microenvironment. These encompass tissue-specific multiple cellularity and architecture, biochemical and mechanical cues, cell–cell and cell-ECM interactions and particularly the three-dimensionality (3D) [48, 49]. Indeed, since the pioneering work of Bissell and colleagues [50], several groups have extensively demonstrated that both normal and transformed cells maintained in traditional 2D culture significantly differ from cells kept in 3D culture in their biological behavior, gene expression profile and drug sensitivity [51–53].

Tissue engineering and regenerative medicine, originally aimed at developing biological substitutes of tissues or whole organs, have been subsequently extended to the generation of 3D platforms attempting to overcome the limitations of conventional culture models [54]. These platforms are based on different approaches, also depending on the aims to be addressed [49]. In particular, several experimental approaches rely on the use of polymeric substrates with tunable composition and stiffness, as scaffolds or hydrogel-based models. Scaffolds are key elements for the generation of 3D platforms, since they provide the mechanical support and physical composition for seeded cells to attach, grow and maintain their specialized functions. A suitable scaffold, such as a bone scaffold, must have favorable biocompatibility or cyto-compatibility and also adequate pore size and interconnectivity, in order to guarantee the growth, differentiation and proper penetration and

distribution of different cell types [55]. Hydrogels are meant to mimic the ECM, and can be either natural or synthetic, the former commonly made with natural polymers (fibrinogen, hyaluronic acid, collagen, Matrigel and gelatin). Synthetic hydrogels are instead typically made with synthetic polymers (polyethylene glycol, polylactic acid, or poly-vinyl acetate) [49].

Scaffold-free models include spheroids and organoids. Spheroids are clusters of cells forced to assemble through hanging drop techniques or culture in bioreactor, taking advantage of the ability of cultured tumor cells to self-aggregate [56]. Spheroids derived from tumor cells, commonly referred to as tumorspheres, are typically monocultures, and therefore lack the multicellular identity that exists in a tumor *in vivo*. Organoids are cell aggregates, whose formation is driven by self-organizing, renewing stem cells, which differentiate *in vitro*, thus reproducing essential aspects of the parental organ [57]. Both structures are being exploited for drug testing, given their suitability for high throughput screening technologies. In particular, organoids grown from patients' tumor tissues (tumoroids) give rise to 3D structures with a multicellular identity that more faithfully recapitulate the complexity of the corresponding tumor they derive from, thus representing an advancement toward personalized medicine [58, 59]. The use of bioreactors and perfused microfluidic chambers adds to the complexity of the culture method, in that it allows a strict control of additional parameters, such as O_2, temperature, pH or nutrients [54]. Finally, the emerging 3D bioprinting technology has attracted increasing attention, based on its potential of manufacturing tissue-engineered compounds with well-defined 3D geometry [60]. In particular, these techniques are used to build tumor constructs via precise injection of living cells (both tumor and stroma) in functional biomaterials (bioinks), thus enabling the spatial–temporal control of molecular physical and chemical gradients [60, 61].

2.3 3D models of multiple myeloma

Since hematological malignancies with BM homing are supported by specialized niches, the complex BM architecture, together with cellular and molecular composition and interactions, needs to be replicated in engineered platforms to reproduce blood cancer behavior [54]. Indeed, while 2D cultures of established MM cell lines have been extensively used in high-throughput drug screening, they fail to reproduce BM microenvironment as well as the heterogeneity of MM patients' cells. The use of primary patient-derived MM cells in 2D monocultures or in co-cultures with stromal cells maintains the heterogeneity of the sample, but MM cell viability and functional interactions are often limited [47]. Finally, several animal models, which have been reviewed elsewhere [62–65], have been developed in order to support the growth of primary myeloma cells within a 3D microenvironment. While these models are more complex and therefore considered as more relevant, they are not representative of the human microenvironment. Within this context, 3D *in vitro*/*ex-vivo* human-derived culture systems are emerging as important tools to generate new approaches to the understanding of the molecular mechanisms of MM progression, essential prerequisites for the development of more effective interventional, diagnostic and prognostic strategies. The former often involve combination of multiple agents with the rationale that combining drugs with different mechanisms and targets could maximize their therapeutic efficacy [11]; this also should be taken into account in the design of 3D models for MM.

Herein we describe relevant 3D models of MM BM microenvironments that were generated exploiting different technical approaches, *i.e.*, gel and solid scaffolds –based 3D platforms, 3D models using microfluidics and 3D constructs cultured in bioreactor.

2.3.1 3D platforms using gel scaffolds

In 2008, Kirshner and co-workers reported the first *in vitro* reconstruction of the human MM BM microenvironment through a 3D model termed "rEnd-rBM". This was achieved by means of a proper overlay of matrix components, specifically collagen I/fibronectin to reconstruct endosteum-marrow junction (rEnd), and then a fibronectin/Matrigel mixture to create the recombinant BM (rBM) compartment, on which isolated cells from BM aspirates of MM patients were seeded [66]. Cells spontaneously redistributed throughout the gel-matrix 3D substrate, mimicking human BM architecture and BM-MM interactions, thus providing a powerful tool for understanding MM biology [66]. Strikingly, the reconstructed BM allowed the expansion of primary myeloma cells, including the putative cancer stem cell fraction embedded within the reconstructed endosteal niche. Moreover, the impact of anti-MM drugs, specifically bortezomib and melphalan, on distinct cellular compartments inside a 3D architecture could be assessed [66].

More recently, de la Puente et al. [67] developed a novel patient-derived 3D tissue-engineered BM culture model complexing BM supernatant of MM patients and autologous cells in a gel scaffold prepared from patient-derived plasma fibrinogen. The resulting construct contained all the growth factors, enzymes and cytokines naturally found in the MM microenvironment of an individual patient, better recapitulating the BM niche. The model reproduced the MM BM hypoxic gradients; moreover, it allowed *ex vivo* proliferation of primary MM cells for several weeks, and induced resistance in MM cells to various anti-myeloma drugs, such as carfilzomib and bortezomib [67].

An additional attempt to mimic the MM niche was performed by Jakubikova and colleagues [68], who developed a new 3D co-culture *ex-vivo* model of primary patient-derived MM cells and BMSC within a commercially available hydrogel (PuraMatrix). BMSC retained phenotypic and functional properties, together with lineage (osteoblastogenic) differentiation capacity. Notably, patient-derived MM cells showed increased proliferation and CXCR4 expression; moreover, BM-driven cell adhesion mediated drug resistance (CAM-DR) to both novel (IMiDs, bortezomib, carfilzomib) and conventional agents (doxorubicin, dexamethasone, melphalan) was observed in the 3D system and paralleled clinical resistance [68].

Finally, a further advancement was reported by Braham *et al* [69], who generated a novel *in vitro* 3D BM niche model by embedding mesenchymal stromal cells (MSC), EC and primary MM cells from patients inside a Matrigel matrix. The model harbored the characteristics of a representative tumor microenvironment, and was able to support long-term (up to 28 days) survival/proliferation of MM cells. The authors successfully exploited this tool to provide the first pre-clinical *in vitro* testing of immunotherapies on primary MM samples inside their tumor microenvironment. In fact, they showed that a novel class of engineered immune cells, *i.e.*, TCRα/β lymphocytes engineered to express tumor-specific Vγ9 VΔ2 TCRs (TEGs) [70], were able to infiltrate the 3D construct and efficiently kill MM cells [69].

2.3.2 3D platforms using silk scaffolds

Adopting a different strategy, based on the use of a strong, porous silk scaffold, MSC were induced to undergo osteogenic differentiation, recreating a mineralized 3D bone matrix [71]. The model allowed to reproduce proper MM-bone interactions in a standardized context and to study the MM-associated osteogenic process, demonstrating the negative impact of myeloma cells on normal bone homeostasis [71]. 3D silk scaffolds have also been employed by the same group to develop the

first 3D, tissue-engineered BM adipose tissue (MAT) model, useful for elucidating the reciprocal interactions between MAT and tumor cells [72].

2.3.3 3D models using microfluidics

Recent technical advances include the tumor lab-on-a-chip, *in vitro* microfluidic devices that provide efficient platforms to recapitulate specific tumor traits, such as angiogenesis, hypoxia and tumor–stroma interactions, thus representing promising tools for personalized medicine [73]. In particular, functional hematopoietic niches have been constructed by culturing and perfusing bone in a sophisticated microfluidic -on-a-chip device [74]. These tools have been exploited to culture MM and BMSC lines, and to investigate MM chemoresistance to bortezomib, as well as the inducible activation of transcription factors [75]; their major limitations rely in the experimental procedure that does not incorporate the interplay between cancer cells the and surrounding stroma, critical to investigate MM progression [76].

2.3.4 3D culture of human MM tissue explants and of isolated MM cells in scaffolds in the microgravity-based RCCS™ bioreactor

The metabolic requirements of complex 3D cell constructs are substantially higher than those needed for the maintenance of traditional 2D cultures under static conditions. To meet this demand, dynamic bioreactors were primarily developed to optimize mass transfer, that is, gas/nutrient supply and waste elimination, all essential factors for preserving cell viability within large 3D cell/tissue masses. Among a wide array of fluid-dynamic bioreactors, the best conditions for long-term culture of functional 3D tissue-like bio-constructs and explants of various origin, including bone, were obtained with the introduction of the microgravity-based Rotary Cell Culture System (RCCS™, Synthecon Inc., USA) bioreactor [77–79] (a vast literature is available at http://www.synthecon.com). On this basis, we successfully employed the microgravity-based RCCS™ technology for the generation and long-term maintenance of viable human-derived MM tissue explants and 3D cell constructs. Our experimental procedure for culturing human tissue samples was firstly validated by using normal (skin and BM) and tumor biopsies [80]. Then, 3D culture of human MM tissue explants was found to maintain overall histo-architecture integrity and viability for up to two weeks. Moreover, the system was suitable for assessing the impact of drugs not only on MM cells, but also on angiogenic vessels, as evaluated through the assessment of MVD [80]. Finally, specialized functions of both MM cells and their microenvironment, including beta-2 microglobulin and cytokine release and metalloproteases activities, could be also assessed [80]. Overall, these observations suggest that 3D culture of MM tissues in bioreactor is feasible and can be potentially exploited as a novel translational tool for patient-specific drug testing.

A major limitation to a systematic pre-clinical use of this approach, however, is represented by the restricted availability of human MM biopsies for tissue culture, besides those obtained for diagnostic purposes. To overcome this limitation, we have recently established a novel procedure based on the reconstruction of a 3D surrogate MM BM microenvironment [81]. This model relies on the co-seeding of MM cells and stroma inside a gelatin sponge, which is subsequently cultured in bioreactor. **Figure 1** schematically represents the procedure developed to generate MM BM surrogate microenvironments, as well as the information that can be obtained through the analysis of both repopulated scaffolds and culture supernatants. Myeloma cell lines engaged contacts with stromal cells, EC and osteoblasts, as assessed by histochemical and electron-microscopic analyses. Consistently,

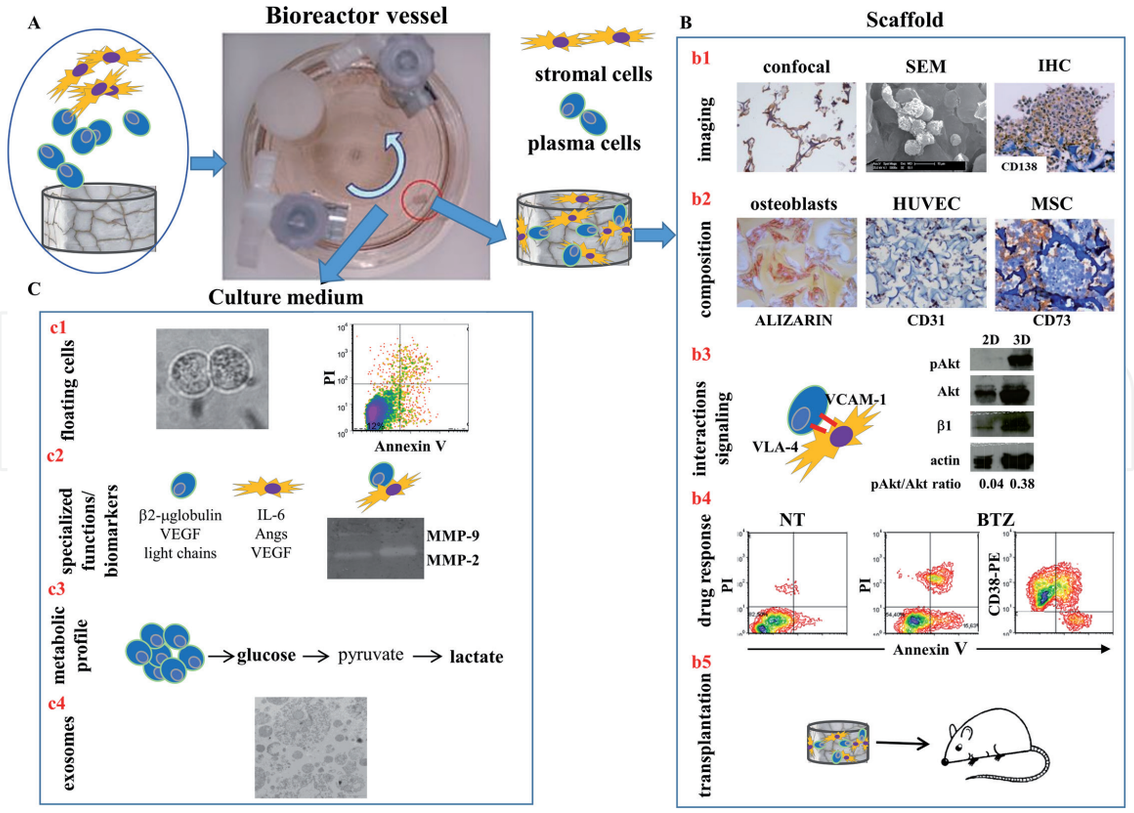

Figure 1.
Information obtainable from 3D MM- BM stroma co-culture in bioreactor. (A) Schematic representation of the experimental procedure: Selected elements of the BM milieu (as in b2, along with lineage-specific markers) and plasma cells are sequentially seeded into the scaffold and kept in culture in bioreactor. (B) Scaffolds retrieved from the bioreactor at the end of the culture period can be: b1: Either fixed or frozen and submitted to imaging by confocal, scanning electromicroscopy (SEM) or immunohistochemistry (IHC) analyses; b3: Lysed and processed for Western blot analysis (right) for the expression of integrins and signaling pathways resulting from tumor-stroma interactions, schematically represented in (left); b4: Enzymatically dissociated to single cells for quantification, characterization and assessment of drug-induced apoptosis by FACS analysis; b5: Ectopically transplanted into mice. (C) Culture medium withdrawn from the bioreactor can be processed to: c1: Characterize floating MM cells, reminiscent of circulating MM cells; c2: Assess specialized functions, attributable to both stroma and MM cells; c3: Determine the content of glycolitic metabolites; c4: Quantify and characterize the content of exosomes. Abbreviations: VEGF = vascular endothelial growth factor; IL = interleukin; Ang = angiopoietin; MMP = matrix metalloproteases; HUVEC = human umbilical vein endothelial cells; MSC = mesenchymal stromal cells; pAkt = phospho-AKT; β1 = β1 integrin; PI = propidium iodide; PE = phycoerythrin; BTZ = bortezomib.

pro-survival signaling and also CAM-DR, particularly through the engagement of the integrin VLA-4 by its counter-receptor VCAM1 [82], were significantly higher in 3D than in 2D parallel co-cultures. Soluble factor-mediated drug resistance could be also appreciated in 3D co-cultures. The system was then successfully applied to co-cultures of primary myeloma cells-primary myeloma BMSC and EC, allowing the functionalization of myeloma-stroma interactions and MM cell long-term survival. Finally, the impact of bortezomib on myeloma cells and on specialized functions of the microenvironment could be evaluated. Significantly, the model also showed the potential for assessing clonal evolution *ex-vivo*. In fact, MM cells obtained from a high-risk patient actively proliferated in bioreactor, paralleling the elevated proliferation index observed in the patient's bone biopsy, and anticipated the expansion of a clone that ultimately dominated *in vivo,* thus predicting the clinical outcome [81].

Further studies validated the use of the model for additional purposes, including investigation on novel pathogenic interactions and preclinical drug testing. In particular, modeling the interaction between the receptor tyrosine kinase ROR2 and its ligand WNT5A in bioreactor allowed identifying this pathway as crucial in the

adhesion of MM cells to the BM microenvironment, and as a potential therapeutic target for the large subgroup of MM patients whose cancer cells show ROR2 overexpression [83]. Moreover, the use of surrogate MM microenvironments in bioreactor complemented studies performed both *in vitro* and in animal models to exploit the DNA damage response as a novel therapeutic strategy for MM. In particular, the combination of drugs causing ATR inhibition (the compound VX-970) and melphalan, a widely used alkylating agent eliciting inter-strand cross-links, proved dramatically effective, thus paving the way to future clinical testing [84]. An additional advantage provided by culture in bioreactor of MM samples or surrogate MM BM on scaffolds is that the well-preserved material can be frozen to create a biobank suitable to serially test patient-specific sensitivity, as for organoids [59].

Our surrogate BM microenvironment could also be exploited for other hematological malignancies infiltrating the BM niches. In particular, Chronic Lymphocytic Leukemia (CLL) is characterized by a progressive expansion of clonal CD5+ B lymphocytes that dynamically traffic from PB to the more protective BM and secondary lymphoid organs, where they acquire an aggressive phenotype and drug resistance [13]. In this context, new targeted therapies, namely kinase inhibitors (KI), have been developed to promote mobilization of leukemic cells from the hosting tissues into the PB, where they may re-acquire sensitivity to drug-induced apoptosis [85]. Our 3D surrogate BM microenvironment was exploited to recreate the niche-specific interplay involved in CLL cells homing/mobilization, showing that distinct molecular interactions, in particular through the HS1 cytoskeletal protein, were reproduced [86]. We could demonstrate that HS1 conversion from the active to the inactive form, promoted by the KI ibrutinib, was able to regulate CLL cells retention inside- and mobilization from- scaffolds. This indicates that the model may serve as a good platform to unveil the mechanisms underlying tumor cells dissemination and to predict the impact of mobilizing agents [86], conceivably also in MM.

The same *in vitro* 3D dynamic culture system in RCCS™ bioreactor was used by Bonomi et al. [87] to generate spheroids of myeloma cells co-cultured with BMSCs. By that mean, the authors demonstrated that BMSCs loaded with Paclitaxel (PTX) could serve as a 'Trojan horse' to vehicle and deliver *in situ* anti-tumor agents in amounts sufficient to affect tumor growth. The inhibitory activity of PTX-primed BMSCs was comparable to that of PTX alone, showing that the loaded-BMSC strategy could be exploited to deliver drugs into the BM.

3. Conclusions and perspectives

The BM, where MM cells home, survive and accumulate, represents a complex and highly specialized tumor microenvironment, making the development of engineered 3D platforms of MM a challenging task. Indeed, in addition to a series of distinct ECM and cellular components, the BM microenvironment comprises several factors, including specialized niches, hypoxic gradients, vascularization and a mineralized matrix, all to be taken into account to faithfully recapitulate the native tumor. Nevertheless, already available pre-clinical models of MM represent a remarkable example of translational cancer research [88], potentially covering issues ranging from high- throughput drug assessment/screening to investigation on MM pathophysiology and patient-tailored drug testing aimed at precision oncology. **Table 1** summarizes the main features of the previously described 3D models of MM BM microenvironments, together with their suitability, in our view, to different purposes. In particular, microfluidic systems could be exploited for drug screening/development with high-throughput potential, in that they can be miniaturized to cope with the limited biological starting material

3D model	Reference	Composition	Drug tested	Drug screening	Precision Oncology	MM biology
Gel scaffold	Kirshner et al., 2008 [66]	ECM components + MM BM aspirates	Melphalan, Btz	—	+	+
Gel scaffold	de la Puente et al., 2015 [67]	Fibrin gel + MM cells + BMSC/EC	Btz, Cfz	—	+	+
Gel scaffold	Jakubikova et al., 2016 [68]	PuraMatrix hydrogel +primary MM cells + BMSC	iMiDs, Btz, Cfz DOXO, DEX, Melphalan	—	+	+
Gel scaffold	Braham et al., 2018 [69]	Matrigel+ BMSC +EC + MM cells	TEGs (□□ T cells expressing V□9 V□2 TCRs)	—	+	+
Solid scaffold	Reagan et al., 2014 [71]	Silk scaffold+ MM cells +BMSC/EC	Btz	—	+	+
Solid scaffold	Fairfield et al., 2019 [72]	Silk scaffold+ MM cell lines+ BMSC	none	—	—	+
Microfluidics	Young et al., 2012 [75]	Microchambers +MM cell lines +BMSC	Btz	+	+	—
Bioreactor-based	Belloni et al., 2018 [81]	Gelatin scaffolds populated by MM cell lines/primary MM cells + BMSC/EC/OB cultured in bioreactor	Btz, Melphalan, DEX	—	+	+
Bioreactor-based	Bonomi et al., 2017 [87]	Spheroids of MM cell lines + BMSC cultured in bioreactor	Paclitaxel	—	+	—

Abbreviations: ECM = extracellular matrix; MM = Multiple Myeloma; BM = Bone Marrow; BMSC = BM stromal cells; EC = endothelial cells; OB = osteoblasts; Btz = bortezomib; Cfz = carfilzomib; iMIDs = immunomodulatory drugs; DOXO = doxorubicin; DEX = dexamethasone.

Table 1.
Summary of different experimental approaches to model the MM BM microenvironment: Potential applications.

than can be obtained from MM patients' samples. Most of the 3D platforms can be used in principle to test a selected range of drugs in a more precise microenvironmental context, in the perspective of personalized therapy and prediction of resistance. Finally, complex 3D technologies, such as bioreactor-based dynamic culture systems, while less easy to handle, can be tuned to recreate proper MM milieu and interactions, thus being suitable to investigation on MM pathophysiology and the mechanisms of drugs. Future efforts combining interdisciplinary basic and technical proficiencies, in particular related to tissue engineering, new biomaterials and advanced imaging techniques [48, 89], are expected to generate fully-humanized, simple, cost-effective, reliable and standardized models that can be more widely employed in the pre-clinical setting, particularly in high-risk and in relapsed/resistant MM patients.

In addition to the purpose of precision oncology, 3D platforms can be applied to explore novel pathogenic cues. In particular, several matters that are object of intense investigation, including hypoxia and tumor metabolism, as well as the contribution of exosomes and miRNAs in the interactions between tumor and its co-evolving microenvironment, could be fruitfully and more precisely investigated applying advanced technological approaches, as already done in different settings (**Figure 1** and [90, 91]). Further exploitation of the SCID/scaffold model, based on the transplantation of 3D bone-like polymeric scaffolds into immunocompromised mice, can also be envisaged to dissect biological events in primary MM cells engrafted inside a human BM microenvironment, as well as their response to drug in a *in vivo* context (**Figure 1B**). Additional future directions include the development and implementation of new technologies, such as microfluidic and bioprinting techniques, to further add to the complexity of *in vitro* surrogate MM BM microenvironments, particularly with regard to MM associated angiogenesis and components of the immune system.

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Conflict of interest

The authors declare no Conflict of Interest.

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