Chapter 4

Heterogeneity and Plasticity of Multiple Myeloma

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1. Introduction

Modern molecular and cytogenetic approaches have furthered progress in our understanding of MM biology and have led to the development of targeted therapy that has improved management of this incurable disease. Novel agents such as bortezomib, lenalidomide or thalidomide, have increased median survival rates and improved prospects for MM patients resistant to conventional therapy [1, 2]. Despite these therapeutic advances, MM remains a very difficult disease to treat still accompanied by the threat of repeated relapses with a fatal ending. These observations indicate that at least some of the MM cells are not targeted efficiently by current drug therapies. The existence of such persistent populations, called myeloma stem cells (MSC) or myeloma-initiating cells (MIC) has been suspected for more than two decades. However, the cells of origin remain elusive [3-9]. Timeline of growing knowledge about putative MSC is displayed in **Figure 1**. Discrepancies among myeloma stem cell concepts have arisen in parallel with the high phenotypic heterogeneity of clonal PCs that might be another factor contributing to the failure of therapies and identification of the population responsible for relapse. Myeloma PCs strongly depends on the supportive role of the bone marrow (BM) microenvironment (MEV) – it is a source of essential growth factors, supports survival and dissemination of pathological PCs [10-14]. Furthermore, hypoxic conditions of tumor microenvironments support tumor progression by inducing angiogenesis, maintaining the malignant phenotype and stimulating osteoclastogenesis [15-18]. There is growing evidence that signals from pathological microenvironments can (reversibly) alter the phenotype of PCs. Such plasticity of PCs might result in obvious heterogeneity of MM and generate inconsistencies among myeloma stem cell concepts.

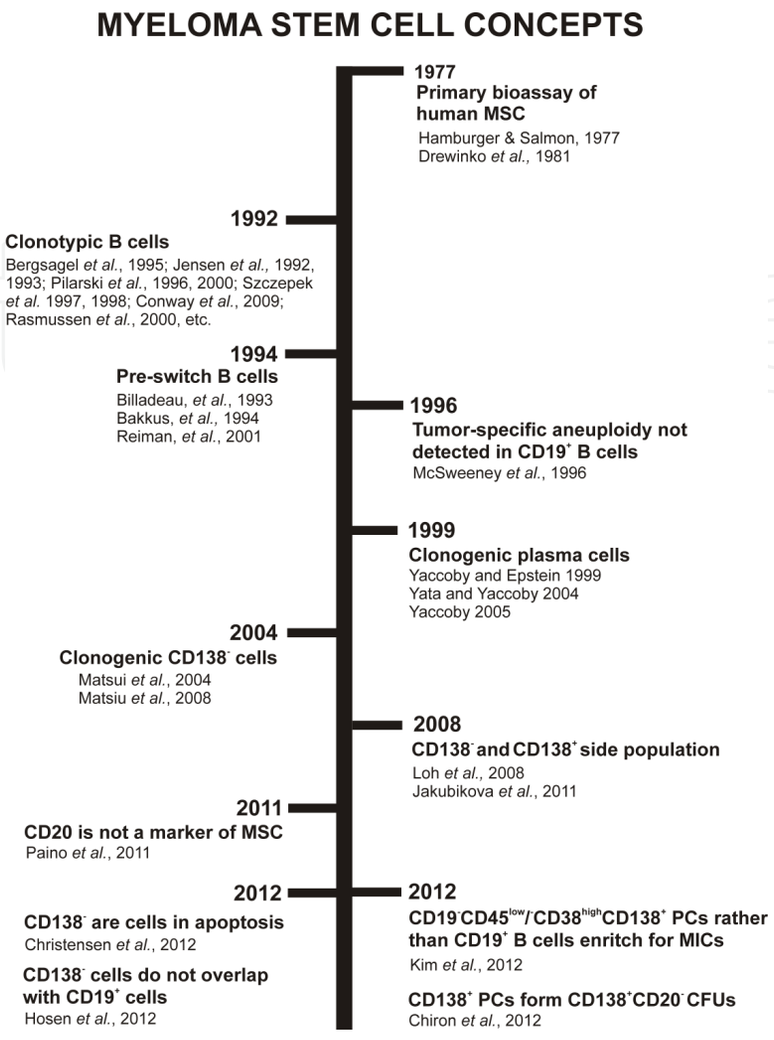

Figure 1. Timeline of myeloma stem cell concepts

2. Myeloma stem cell concepts

A number of laboratories have tried to identify a biologically distinct population of so called myeloma precursors or myeloma stem cells (MSC) which are responsible for the incurability of MM (see **Table 1**). However, none of these concepts have been unambiguously proven until now. With regards to the fact that abnormal PCs of MM show features of advanced differentiation and mature morphology, the population responsible for the origin and sustainability of tumor mass has been suspected in the minor population of clonotypic or clonogenic $CD138^-$ cells retaining key stem cell properties, tumor-initiating potential, self-renewal and resistance to chemotherapy [6, 19-25]. However, it was demonstrated that even the dominant population of human $CD138^+$ PCs contained clonogenic cells; these cells show plasticity potential that might be responsible for dedifferentiation and acquiring of stem cell properties [8, 26-30].

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	Characteristics of the cells	Citation
Clonotypic B cells	PB CD19, CD38, CD10, CD11b, CD34 (HPCA-1), variable CD20, PCA-1, CD45RO, variable CD45, and CD56	[19-23, 31-34]
	PB CD34$^{\pm}$, CD38^{+}, CD184^{+}, CD31$^{\pm}$, CD50$^{\pm}$, CD138^{-}, CD19^{-}, CD20^{-}	[35]
	PB CD19^{+}CD27^{+}CD38^{-} memory B-like cells	[24]
Clonotypic pre-switch B cells	pre-switch somatically hypermutated clonotypic cells (VDJ sequence still joined to Cμ gene) PB and BM CD19, HLA class II or surface IgM	[36-38]
Clonogenic CD138^{-} cells	BM CD20^{+}CD27^{+}CD34^{-}CD138^{-} clonogenic myeloma stem cells	[6, 39-41]
Side population	capacity to exclude dyes sideways from the diagonal in FACS analysis plots CD138^{-} and CD138^{+}	[42, 43]
Clonogenic plasma cells	BM CD38^{+}CD45^{-} PC (SCID-hu model) BM clonogenic CD38^{+}CD138^{-}CD45^{-} PC (SCID-rab model)	[7, 26, 28]
	BM CD38^{+}CD138^{-}CD45^{-}CD19^{-}CD34^{-} PC (osteoclast coculture)	[27]
	CD19^{-}CD45$^{low/-}$CD38^{high}/CD138^{+} PCs	[30]
Intermediate PC precursors	CD138+CD44+ give rise to short-lived PC CD138+CD44-/CD138-CD44+ give rise to long-lived PC in murine model candidates for the normal counterpart of transformed MM cells	[44-46]

Table 1. Candidates for putative neoplastic PC precursors/MSC.

2.1. Clonotypic B cells

Myeloma precursors that share identical variable diversity joining (VDJ) regions, rearrangements of immunoglobulin heavy chain gene (*IgH*) with patient's tumor PCs and show a pre-plasma cell phenotype are referred to as clonotypic B cells (CBL). They have been identified in peripheral blood, lymph nodes and bone marrow [32, 36, 37, 47-49]. An extensive accumulation of somatic mutations in *IgH* gene and an absence of intraclonal variation suggest that CBLs originate from post-germinal center B cells [37, 50]. Although the concept of clonotypic B lymphocytes as neoplastic PC precursors is widely accepted, other studies suggest the existence of MM precursors in other compartments (see Table 1). Additionally, phenotypic profile and amount of CBLs vary among studies – e.g., it is not clear if these cells resemble $CD19^{+}CD27^{+}CD38^{-}$ memory B-cells or carry a marker of hematopoietic stem cells, CD34 with or without expression of the CD19 surface marker [21, 23, 35]. Furthermore, McSweeney *et al.* (1996) did not find bone marrow $CD19^{+}$ cells to be clonally restricted to kappa/lambda or revealed any deviations of expression patterns of B-cell maturation markers from normal B-cell components [51]. His results do not support a hypothesis of disturbed B-cell maturation and development of the disease from early stages of B-cell ontogeny. Moreover, DNA analysis proved that $CD38^{++}CD19^{-}$ cells were aneuploid in most cases with a typical cell cycle profile indicating the presence of a proliferating population, while cells expressing CD19 were diploid. This suggests the existence of self-replicating plasma cell compartments that have a capacity to replenish the tumor without the involvement of early B lymphocyte progenitors.

A number of studies that confirmed the presence of clonotypic B cells have been based mainly on the detection of the same IgH rearrangements by PCR-based methods. The percentage of MM patients with IgH-positive clonotypic B-cells ranged from 40% and 87% [36, 48, 52]. Limited dilution PCR assays detected the abundance of CBLs in 0.24% - 25% of peripheral blood mononuclear cells (PBMC) and 66% of all peripheral B cells [32, 36, 52, 53]. This wide range of occurrence may result from methodological errors of the PCR technique or expression of atypical, non-clinical Ig transcripts [53]. The major disadvantage of the PCR method is that this approach does not allow the morphological identification of cells of interest or sort cells for subsequent functional analyses. In addition, it is important to take into account that the presence of the same IgH rearrangements is reliable marker of clonality; it does not confirm the malignity of cells *per se*. Therefore, the detection of IgH rearrangements in $CD19^{+}$ cells could also indicate that a small clone of premalignant or premyelomatous B cells persists despite the transformation of other cells into malignant clones but this population does not actively contribute to myeloma clones [51]. Rasmussen *et al.* (2010) suggests that peripheral blood memory B cells represent pre-malignant, partially transformed remnants that could have some proliferative advantage over normal memory B cells. They questioned the involvement of CBLs in MM maintenance due to the fact these cells expressed specific 'early' oncogenes (*FGFR3, MMSET, CCND1*) that were deregulated by an IgH translocation but lacked 'late' oncogene (*KRAS*) mutations [54].

A large interpatient and interstudy variability led Trepel *et al.,* (2012) to establish patient-individual ligands mimicking the epitope recognized by the myeloma immunoglobulin to specifically target clonotypic surface IgH-positive B cells of MM patients [53]. In a cohort of 15

MM patients, semi-nested PCR using HCDR3-specific patient-individual primers detected CBLs in 50% of peripheral blood and/or bone marrow samples. This frequency is line with published data. However, a new flow cytometric protocol detected clonotypic B cells only in one patient with a sensitivity of 10^{-3}, ie. less than one clonotypic B cell per 1000 PBMCs. These cells accounted for about 0.15% of PBMCs and 5% of B cells in this patient. Surprising discrepancies between these two approaches could indicate nonspecific annealing of the CDR3-primer leading to false positive PCR results. Conclusions of this study suggest that the abundance of CBLs is exceedingly low and has been enormously overestimated in previous studies. Similarly to findings of Rasmussen *et al.* (2010), authors consider that unlike those CBLs they are true "feeder" cells for malignant PC compartments and an essential prerequisite of myeloma maintenance and progression [54]. In regard to the rare occurrence of CBLs in MM, it is more likely that these cells are non-malignant remnants, which may be a part of the malignant plasma cell clone but do not participate in tumor maintenance [53].

2.2. Clonotypic pre-switch B cells

An identical rearrangement of the IgH VDJ region with a consistent pattern of hypermutations within the clone is a signature of CBLs. MM plasma cell clones generally express monoclonal Igs of these isotypes: IgG, IgA, IgD, IgM, in rare cases kappa or lambda light chains and non-secretory myeloma is detected [55]. Clonotypic isotypes with identical Vh gene sequences and patterns of hypermutations linked to different classes of Ig heavy-chain constant region genes (including Cμ gene) compared to clinical isotypes have been identified in some MM patients [36-38, 56]. B cells with "non-clinical" isotype have been called pre-switch (IgM^+) clonotypic B cells. Particular classes of Ig are generated by mechanism of class switch recombination (CSR) resulting in the formation of a hybrid switch region composed of switch region Sμ and a respective isotype switch region (Cδ, Cγ3, Cγ1, Cα1, Cγ2, Cγ4, Cε and Cα2). Consequently, immunoglobulin production is 'switched' from IgM to IgG and IgA (occasionally to IgD or IgE). Sequences between the switch regions are cut out as a deletion loop after double-stranded breaks without changing the VDJ sequence [57-59]. The presence of CSR is a hallmark of "post-switch" B cells. However, pre-switch B cells do not seem to undergo CSR because IgH transcript is composed of the clonotypic VDJ sequence still joined to Cμ gene [56].

Preswitch (IgM^+) CBLs have been identified in BM and PB of most MM patients; however their frequency is very low. Palumbo *et al.* (1992) have already suggested that MM may originate from pre-switch cells [60]. Billadeau *et al.* (1993), using the allele-specific oligonucleotide PCR, demonstrated the existence of the pre-switch isotype species that were clonally related to the myeloma tumor. Another study also proved the evidence of pre-switch cells with somatically hypermutated clonotypic VDJ region [37]. These cells exhibited expression of CD19 and HLA class II or surface bound IgM. Reiman *et al.* (2001) described the presence of clinical and nonclinical clonotypic isotypes in PB, BM and G-CSF – mobilized blood autografts of MM patients. Expression of preswitch clonotypic transcripts persisted in the blood despite high-dose chemotherapy with stem cell support suggesting drug resistance of this pre-switch population. The persistence of preswitch clonotypic isotypes was associated with reduced survival and with a more advanced disease at the time of diagnosis. Moreover, both pre-

switched and postswitched cells were able to engraft in the NOD/SCID mice indicating their potential clinical relevance. Authors considered the fact that MM is the disease of post-switch cells, IgH isotype switching in MM may accompany worsening disease [38]. Despite these facts, Taylor *et al.* (2008) questioned the role of preswitch (IgM^+) clonotypic cells as a progenitor pool for postswitch MM-PCs. They hypothesized that if they are progenitors for MM-PCs, multiple clonotypic switch junctions, or changes in the switch junction, are expected in the postswitch progeny. However, results of specific clonotypic-switch PCR determined the presence of a single, unchanged clonotypic switch junction. Thus, postswitch MM-PCs most likely originate from a single CSR event and pre-switch IgM^+ cells do not represent MM-PC progenitors [52].

Interestingly, pre-switch (non-clonotypic) IgM^+ $CD27^+$ cells with mutated IgH V region have been detected in various immunodeficiencies and autoimmune diseases but also in healthy donors [61, 62]. Expression of surface marker CD27 suggests that these cells maybe IgM^+ $CD27^+$ memory B cells. On the other hand, considering the occurrence of IgM^+ $CD27^+$ cells in humans who cannot form germinal centers (GC), it was presumed that these cells: 1) are generated independently of germinal centers and therefore cannot represent a subset of memory B cells; 2) undergo SHM during generation of the preimmune repertoire; and 3) they mediate responses to T cell-independent antigen. The evidence for these presumptions came from the studies of patients with hyper-IgM syndrome, X-linked lymphoproliferative disease or common variable immunodeficiency that are characterized by some type of defect in GC formation. However, quantification of IgM^+ $CD27^+$ cells in these diseases showed that their numbers reached approximately 20–40% of the number observed in normal individuals. These results indicate that germinal centers may play a role in the development of IgM^+ $CD27^+$ cells. IgM^+ memory B cells may be generated at the centroblast stage and undergo SHM but leave GC before the onset of isotype switching. Whether IgM^+ $CD27^+$ cells with mutated IgH V region might be a source of preswitch (IgM^+) CBLs, it remains a matter of further debate and research.

2.3. Clonogenic $CD138^-$ cells

Matsui *et al.,* (2004) suggested that the source of MSC responsible for the initiation and maintenance of MM might be due to a minor population of less differentiated cells reminiscent of memory B-lymphocytes with surface markers $CD20^+CD27^+CD34^-CD138^-$. They showed that $CD138^-/CD34^-$ cells derived from MM cell lines RPMI 8226, NCI-H929 and primary clinical samples were clonogenic *in vitro.* Cloning efficiency correlated with the disease stage [6]. The depletion of either $CD20^+$ or $CD27^+$ cells from the $CD138^-/CD34^-$ population significantly limited clonogenic growth of MM, therefore the phenotype of MM cells with *in vitro* clonogenic potential were suggested to be characterized by a pattern of surface markers $CD20^+CD27^+CD138^-$ [39]. Clonogenic potential of $CD138^-/CD34^-$ cells was evaluated by successful engraftment of non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice during both primary and secondary transplantation. $CD138^+/CD34^-$ were unable to form colonies *in vitro* and human engraftment was not detected in any of the mice injected with $CD138^+$ cells. A chimeric anti-CD20 monoclonal antibody, rituximab, was showed to

inhibit clonogenic growth of $CD138^-$ cells *in vitro* [6,39]. However, clinical trials failed to confirm an effect of rituximab as a useful maintenance therapy for MM [63, 64].

Paino *et al.*, (2011) reevaluated the presence and function of $CD20^+$ putative MSC in a panel of myeloma cell lines [65]. Although, Matsui *et al.* (2004) described a small population (2-5%) of $CD138^-20^+$ cells in NCI-H929 and RPMI-8226 cell lines, Paino *et al.* (2011) were not able to detect CD20 by flow cytometry in the majority of tested MM cell lines [6, 65]. Only RPMI-8226 cell lines contained a small population of $CD20^{dim+}$ cells (0.3%). These data are consistent with the report of Rossie *et al.* (2010) that showed that U266, NCI-H929 and RPMI-8226 MM cell lines are $CD20^-$ [66]. Despite previous results, memory B-cell phenotype of putative $CD20^+$ MSC was also not confirmed. On the contrary, $CD20^{dim+}$ cells displayed a myelomatous plasma cell phenotype: $CD38^+CD138^+CD19^-CD27^-CD45^-$. Additionally, $CD20^{dim+}$ cells did not exhibit stem cell properties and compared to the $CD20^-$ population they showed a lower level of self-renewal potential. Both populations developed plasmacytomas when they were injected into CD17-SCID mice suggesting that $CD20^{dim+}$ cells are not essential for tumor formation. Furthermore, sorted and plated $CD20^{dim+}$ cells did not differentiate into $CD20^-$ cells. However, $CD20^-$ cells give rise to $CD20^{dim+}$ cells indicating a hierarchical order of differentiation from $CD20^-$ to $CD20^{dim+}$ cells. Overall, these results do not support CD20 as a marker associated with MSC phenotype [65].

Other drugs, such as dexamethasone, lenalidomide, bortezomib, or 4-hydroxycyclophosphamide did not significantly affect $CD138^-$ cells indicating resistance to some conventional or novel therapy, a characteristic feature of CSC [39]. All four agents significantly inhibited the clonogenic growth of $CD138^+$ cells isolated from MM cells. Nevertheless, it could not be evaluated in $CD138^+$ PCs from MM patients because they lack *in vitro* clonogenic activity. Detection of a small $CD138^-$ population of MM cell lines (< 2%) that displayed stem cell properties mediating drug resistance, such as the capacity to efflux the DNA binding dye Hoechst 33342 and higher relative levels of aldehyde dehydrogenase (ALDH) activity further supports the existence of a resistant MSC compartment in MM. Moreover, $CD138^-$ cells of MM cell lines also exhibited cellular quiescence similar to adult stem cells – almost all $CD138^-$ cells were shown to remain in $G_0 – G_1$ phase and less than 1,5 % in S phase, compared to two thirds of $CD138^+$ cells in $G_0 – G_1$ and about 20% in S phase [39]. On the other hand, results of the first study proved that $CD138^-$ cells isolated from the same MM cell lines expressed higher levels of the proliferation marker Ki67 than $CD138^+$ cells [6]. These discrepancies might come from different passages of cell lines or culture conditions but cannot conclusively prove a quiescent state of $CD138^-$ cells.

Besides the clonogenic population of $CD138^-$ cells, Matsui *et al.* (2004) also isolated circulating clonotypic $CD19^+CD27^+$ B cells in peripheral blood of MM patients which were successfully engrafted into NOD/SCID mice. However, it remains unclear whether $CD138^-$ clonogenic cells are identical to clonotypic $CD19^+$ B cells. Hosen *et al.* (2012) examined 16 MM samples and found that only $CD138^-CD19^-CD38^{++}$ cells formed colonies *in vitro* whereas $CD19^+$ B cells did not [67]. Moreover, $CD138^-CD19^-CD38^{++}$ cells engrafted into SCID-rab mouse models in 3 cases out of 9, whilst there was no detection of $CD19^+$ B cell engrafment. Neither $CD19^+$ B cells transplanted into NOD/SCID IL2Rγc(-/-) mice propagated into MM. Surprisingly, $CD138^+$ PCs

also give rise of MM, but more slowly than $CD138^-$ cells. Thus, CD138-negative clonogenic cells might represent a population which has the potential to give rise to MM but does not overlap with the population of $CD19^+$ B cells. These results indicate that CD138-negative clonogenic cells are more PCs than B cells but this does not necessarily mean that Matsui's myeloma stem cell concept is wrong. Although, MSCs phenotypically resemble memory B cells, they may be modified PCs and CD138-positive PCs might represent some "transit" population that subsequently lost its mature phenotype (this will be discussed later). However, Christensen *et al.* (2012) reexamined $CD138^-$ population of so called MSC and obtained surprising results which were strongly controversial to both previous mentioned reports [68]. An analysis of primary $CD138^+$ PCs of MM patients showed that the number of $CD138^-$ cells increased in parallel with increasing time from sampling to analysis. In regards to the fact that myeloma PCs loses expression of the surface antigen CD138 in apoptosis, Annexin V was included in all analyses to monitor apoptotic cells [69]. Expectedly, if a $CD138^-$ population was detected, these cells were positive for Annexin V. Similar results were also obtained by Chiron *et al.* (2011). Furthermore, qPCR techniques confirmed similarly high levels of CD138 mRNA in both $CD138^-$ and $CD138^+$ MM cells. $CD138^-$ and $CD138^+$ subpopulations varied neither in expression of CD19 nor in expression of CD20. These contradictory results would imply that the CD138-negative population may represent only cells undergoing apoptosis as a consequence of previous sample handling [68]. Nevertheless, these new findings cannot completely deny results of previous studies that showed the potential of $CD138^-$ cells to form colonies *in vitro* and engraft mouse models.

2.4. Side population

Side population (SP) cells were defined based on their capacity to exclude dyes such as Hoechst 33342 [70]. Hoechst 33342 dye binds to the AT-rich regions found in the minor groove of DNA. Upon UV excitation, cells with this efflux capacity can be identified as the minor population of the positively stained cells sideways from the diagonal in FACS analysis plots. SP cells have been detected in various cancer cell lines as well as primary tumors (rev. in 71). Side population possesses CSC characteristics such as the capacity for regrowth of the tumor, expression of stem cell-like genes and resistance to chemotherapy. This is why SP cells are believed to be the true population responsible for tumor maintenance.

The presence of SP cells were investigated in four MM cell lines, RPMI 8226, U266, OPM2 and KMS-11, and primary MM samples [42]. SP was defined using control cells stained with both Hoechst and verapamil, L-type calcium channel blocker, to establish the SP gate[72]. Reduction of SP cells was demonstrated in all tested cell lines. In 18 of 21 bone marrow samples from MM patients the percentage of SP cells ranged from 0 to 4.9% compared to 0.05% of SP in normal bone marrow. There was neither a significant difference between treated and non-treated MM patients based on the percentage of SP nor a correlation between the percentage of SP and the paraprotein concentration or disease stage. 0.18–0.83% of SP cells express the clonal surface immunoglobulin light chain restriction that matched 89–97% of each patient's PCs. SP cells were also analyzed for the expression of CD138. A mean of 96.1% of SP cells were found to be $CD138^-$. However the $CD138^+$ fraction also contained SP indicating that they are present in

both $CD138^-$ and $CD138^+$ compartments. Jakubikova *et al.* (2011) also demonstrated that the SP fraction of MM cell lines expressed CD138 antigen [43]. Moreover, these results did not prove a correlation between expression of CD19, CD20, or CD27 and the proportion of SP cells. Conversely, SP cells showed more clonogenic potential and proliferation index than the main population. Furthermore, adherence to stromal cells increased percentage, viability and proliferation potential of SP cells. Supportive role of BM microenvironment was attenuated by lenalidomide and thalidome. Lenalidomide itself directly decreased the percentage and clonogenicity of SP cells. This study demonstrated innovative and promising strategies for targeting putative myeloma-initiating cells and prevention of relapse.

Although SPs exhibit stem cell properties and might represent a "feeder" population responsible for the relapse of the disease, others question the method for detection of SP. Hoechst staining binds to DNA resulting in toxicity to live cells, that is why SP cells might represent only a population that survived the lethal effect of Hoechst. SP phenotype might also be affected by staining time, dye concentration or cellular concentration. The problem also lies in cytometric approaches that showed inconsistencies among gating strategies and might lead to contamination of the SP fraction by non-SP cells. Furthermore, usage of verapamil as an inhibitor of efflux was also criticized because verapamil-sensitive cells were detected in the negative or SP gate [73]. These problematic findings require more stringent gating strategies to clear doubts about SP.

2.5. Clonogenic plasma cells

The clonogenic potential of primary MM cells was first demonstrated by Hamburger & Salmon (1977). They showed that freshly explanted human myeloma cells are able to form colonies of monoclonal PCs. Colonies consisted of immature plasmablasts and mature PCs. Drewinko *et al*., (1981) investigated the growth fraction of MM cells. In untreated and nonresponsive patients the growth fraction represented 4% of MM cells. Patients in relapse had the growth fraction ranging from 14% to 83%. Nonproliferating fraction contained true quiescent cells, some proliferating cells with very long intermitotic times, and some proliferating cell that have entered the maturation phase. Although these two reports demonstrated clonogenic and proliferative capacity of primary MM tumors, these cells were not phenotypically defined as whole mononuclear fraction of BM was used for analyses. Therefore, results of these studies cannot answer what kind of cells represents true growth fraction.

First experiments demonstrating the clonogenic growth of phenotypically defined PCs were carried out by Yaccoby and Epstein (1999). They proved that $CD38^{++}CD45^-$ PCs derived from PB and BM are able to engraft severe combined immunodeficiency (SCID)-hu host system with implanted human bone. Circulating clonal PCs grew more rapidly in SCID-hu hosts than those in the BM suggesting that this may represent a subpopulation with a higher growth potential. In contrast to previous reports, PC-depleted blood cells did not give rise to MM in SCID-hu hosts [7]. Yata & Yaccoby, 2004 presented an alternative model for study of myeloma-initiating cells (MIC) that uses rabbit bones implanted subcutaneously in unconditioned SCID mice. The SCID-rab model was also successfully engrafted with $CD138^+$ PCs of MM patients. Although, these two models did not show a serial engraftment of the disease, they strongly indicate a

critical role of specific bone marrow microenvironments for plasma cell survival and proliferation [28].

A dependence of myeloma PCs on human BM microenvironment was clearly demonstrated in an experiment with NOD/SCID/common cytokine receptor γ chain-deficient (NSG) and recombinase-activating gene 2/common cytokine receptor γ chain-deficient (RAG2-/ γ c-) mice [30]. Results proved that $CD138^{+}/CD38^{high}$ cells from MM patients led to a repopulation of $CD19^{+}CD38^{low}$ or $CD138^{+}CD38^{+}$ B-lineage cells in human bone-bearing mice but no engraftments were detected in human bone-free mice even after orthotropic intrafemoral injection. Moreover, serially xenotransplantated $CD19^{-}CD138^{+}$ cells preferably engrafted from a human bone graft but were not detected in any mouse hematopoietic tissues. All grafts derived from $CD138^{+}/CD38^{high}$ cells were clonally related to myeloma PCs, whereas engraftments of $CD19^{+}CD38^{low/-}$ B cells were polyclonal $CD19^{+}CD38^{low}$ cells. Further fractionation of $CD138^{+}/CD38^{high}$ cells and theirs subsequent xenotransplantation showed that $CD45^{low/-}$ or $CD19^{-}CD38^{high}/CD138^{+}$ cells had a higher engraftment ability than $CD45^{high}$ or $CD19^{+}$ plasmablasts. In line with the above mentioned results, it was concluded that $CD19^{-}CD45^{low/-}CD38^{high}/CD138^{+}$ PCs rather than $CD19^{+}$ B cells or plasmablasts enrich for MICs. In addition, analysis of clonogenic potentials of $CD138^{+}$ and $CD138^{-}$ populations derived from plasma cell leukemia patients strongly favored these findings [29]. This study demonstrated that, though, $CD138^{+}$ PCs derived from plasma cell leukemia patients formed colony forming units (CFU) in a very low frequency, no CFU were found when $CD138^{-}$ cells were seeded. Harvested cells of CFUs derived from $CD138^{+}$ PCs were strongly positive for CD138 but negative for CD20. There was no evidence that they can be $CD138^{-}CD19^{+}CD20^{-}$ plasmablasts or $CD19^{+}CD20^{+}CD138^{-}$ B-cells. These findings further underline an important role of $CD20^{-}CD138^{+}$ population as a conceivable reservoir of clonal PCs at least in plasma cell leukemia.

Against the statement that CD138-positive PCs represent a "feeder" population responsible for incurability of MM is the fact that abnormal PCs show a low proliferation potential with a plasma cell labeling index (PCLI) ranging from 0.5% in MGUS to 1% in early MM, PCLI. Therefore, the growth fraction of progenitor cells giving rise to MM has been expected in less differentiated stages of B cell development. Despite low proliferative activity of malignant PCs, PCLI is one of the most important prognostic factor with a strong impact on overall survival [98, 99]. Additionally, limited proliferation potential of PCs could be explained by error reduction during DNA synthesis that was proposed for normal adult stem cells that are highly clonogenic but proliferatively silenced [100].

2.6. Intermediate plasma cell precursors

O'Connor *et al.* (2002) identified post-GC-precursors in a mouse model that might contribute to long-lived humoral immunity. These cells were distinct from splenic B cells, mature or memory B cells as well as mature PCs. Mediate levels of CD138 indicated that PC precursors might represent a transition state of PC development. PC precursors were demonstrated to migrate to the BM where they proliferate and persist for a long period of time and consequently differentiate to mature PCs without antigen stimulation. $CD138^{+}CD44^{+}$ give rise to short-lived PCs, $CD138^{+}CD44^{-}$ or $CD138^{-}CD44^{+}$ differentiated to long-lived PCs. Two roles of PC precur-

sors were suggested; (i) they might either serve as a reservoir of PCs upon PC attrition or (ii) contribute to post-GC affinity maturation of the humoral immune response. Due to similarities of phenotype, proliferative potential, and differential capacity of the PC precursors to putative MM progenitors, authors suggested that these cells are candidates for the normal counterpart of transformed MM progenitors [74].

The true origin of MM remains a matter for further debate. An unanswered question persists whether clonotypic $CD138^{-}$ cells or side populations represents myeloma precursors or phenotypic variants of the MM tumor cells that have lost some plasma cell markers and gained some B-cell markers as Yaccoby (2005) suggested [27, 54]. He presented a new model for myelomagenesis; that mature $CD45^{low/intermediate}$ $CD38^{high}$ $CD138^{high}$, $CD19^{-}CD34^{-}$ PC situated closely with osteoclasts in the lytic bone lesions reverse senescence, acquire a stem cell phenotype, ($CD45^{intermediate/high}$ $CD19^{low}$ and$CD34^{low}$), and become quiescent and apoptosis-resistant. This indicates that myeloma PCs have plasticity that allows them to reprogram, dedifferentiate and acquire autonomous survival properties which are responsible for drug-resistance and relapse of MM patients. Similar results were also obtained by Kukreja *et al.* (2006) when cultured U266 myleoma cell line and primary myeloma PCs with dendritic cells (DC). The coculture of U266 cells and DCs led to an increase in the proportion of cells lacking CD138; a marker of terminally differentiated PCs, and induction of B cell lymphoma 6 (BCL6) expression. It was suggested that BCL6 plays an important role in survival and self-renewal of germinal center B cells and that suppression of BCL6 is a critical feature of normal PC differentiation. Furthermore, a presence of DCs in the culture enhanced clonogenic growth of the myeloma cell line and as well as primary myeloma PCs [75]. Just as in the previous report, this data suggests that the differentiation state of myeloma cells is plastic and can be modified in the presence of DCs. Both reports emphasize an importance of tumor microenvironments for myleoma plasticity. Whether myeloma PCs exhibit plasticity that might be responsible for phenotypic heterogeneity of MMs, and also form inconsistencies among myeloma stem cell concepts, it will be discussed further.

3. Phenotypic heterogeneity of multiple myeloma

The high phenotypic heterogeneity of clonal PCs is a hallmark of MM; abnormal PCs frequently express a wide spectrum of multi-lineage antigens such as myeloid, T-cell and natural killer-associated antigens. Compared to normal polyclonal PCs which show high CD38 and CD138 expression along with the B-lymphocyte marker CD19 abnormal PCs generally lack CD19 expression, show variable expression of CD45, dim expression of CD38 and heterogeneous signals of CD138. Further, they have weaker expression of CD27, increased expression of CD28, CD33 and CD56 and variable expression of CD20 and CD117 (rev. in [76, 77]). Causes of phenotypic heterogeneity remain unclear.

PCs belong to the B-cell lineage that expresses paired box protein 5 (PAX5) and its target, CD19. PAX5 activation and inactivation is essential for early B cell commitment as well as for maintenance of the functional identity of B cells throughout B cell development. PAX5

represses B lineage 'inappropriate' genes and simultaneously activates B lineage-specific genes [78, 79]. Conditional PAX5 reduction in late B lymphocytes promotes development to the mature B cell stage. PAX5 and CD19 expression are considered to be downregulated or lost but it has been reported that expression of PAX5 and CD19 is restored in normal PCs of pleural effusion, ascitic fluid and BM aspirate. In contrast to normal PCs, clonal PCs of MM do not express PAX5 or CD19 (> 95% of cases) [80, 81].

Phenotypic heterogeneity of abnormal PCs might reflect a certain degree of dedifferentiation of these cells. It has been previously shown that elimination of PAX5 shifts B cells to multipotency [82]. Therefore expression of multi lineage markers on clonal PCs might be due to a lack of *PAX5* gene expression but not only as a consequence of malignant transformation. Cell heterogeneity is the phenomenon that is commonly seen in many cancer types. The cause of this variability might be due to the loss of the lineage master gene which can lead to dedifferentiation or transdifferentiation into cells of other lineages [83].

The ability to differentiate into multiple lineages is a characteristic feature of pluripotent embryonic stem cells. However, pluripotency does not necessarily have to be limited to a population of undifferentiated stem cells of the early embryo and lost irreversibly upon terminal differentiation [84]. Fully differentiated somatic cells can be reprogrammed into inducible pluripotent stem cells (iPSC) by 'forced' expression of pluripotency/reprogramming factors: OCT4, SOX2, c-Myc and KLF4 or NANOG, LIN28, c-Myc and KLF4 [85, 86]. Human somatic cells from all three germ layers, including human differentiated mature B lymphocytes, have been successfully reprogrammed [87-93] Reprogramming of mature B cells required additional 'sensitization' by the myeloid transcription factor CCAAT/enhancer-binding protein-α (C/EBPα) that causes a disruption of PAX5 functions, or a specific knockdown of the B cell transcription factor PAX5. This indicates that the loss of PAX5 expression might be associated with gain of stem cell features for mature PCs and represent one of the key events in the pathogenesis of MM.

4. Plasticity of multiple myeloma

There is growing evidence that MM encompasses a certain degree of plasticity that might be responsible for expression of a wide spectrum of multi lineage markers and discrepancies among myeloma stem cell theories. Two facts support this hypothesis:

i. There is a strong link between induction of pluripotency and tumor progression; several human cancers acquire stem cell-like plasticity upon (re)expression of reprogramming factors. It raises the possibility that dedifferentiation is a key mechanism for the generation of tumor-initiating cells in human cancer [84]. The process of dedifferentiation is suggested to be under control of tumor microenvironment. Interaction of tumor cells with their microenvironment might induce altered differentiation; epithelial-mesenchymal transition (EMT) which has been observed in some solid tumors [94, 95]. Similarly to MM, phenotypic plasticity of myeloma PCs were observed in long-term co-culture with osteoclasts; myeloma PCs lost their

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mature phenotype and dedifferentiated to an immature, resilient, apoptosis-resistant phenotype. In addition, $CD138^+$ PCs cocultured with DCs lost expression of CD138 and increased the clonogenic potential [75]. It raises the possibility that signals from the microenvironment can (reversibly) alter the phenotype of PCs and cause obvious heterogeneity of MM. This regulation might keep dynamic equilibrium between CSC and non-CSC compartments [96].

Dynamic state between CSC and non-CSC like compartments were also observed in a subpopulation of differentiated basal-like human mammary epithelial cells that spontaneously converted to stem-like cells *in vitro* and *in vivo*. Moreover, oncogenic transformation enhances this spontaneous conversion. These findings indicate that normal and CSC-like cells can arise *de novo* and indicate the importance of the differentiation state of cells-of-origin as a critical factor determining the phenotype of their transformed derivatives [97]. Whether mature PCs exhibit a plasticity potential responsible for dedifferentiation into cells with stem cell like properties is questionable but highly attractive. Confirmation that mature PCs have the capacity to convert into CSCs could resolve current inconsistencies among myeloma stem cell theories and help to target the population responsible for relapse of the disease.

ii. Despite of mature phenotype, myeloma PCs have been reported to express pluripotency factors (SOX2, c-Myc or KLF4) and stem/progenitor markers, such as germline stem cell markers of the MAGE family, the hematopoietic progenitor marker CD117 or neural stem cell marker nestin [83, 101-105]. Some of these factors provide valuable prognostic information. Currently, the role of c-myc is growing as the key transforming factor in the progression of asymptomatic MGUS to a symptomatic disease [101]. SOX2 and MAGE have been demonstrated to be relevant targets for immunotherapy due to their immunogenicity. Interestingly, MGUS patients frequently mount a humoral and cellular immune response against SOX2 but MM patients lack anti-SOX2 immunity [104, 106]. CD117 (c-kit) is an essential hematopoietic growth factor receptor with tyrosine-kinase activity. Aberrant expression of CD117 is detected on a subset of MGUS and MM and is associated with a favorable outcome for MM patients [107, 108]. KLF4 was described in regulation of apoptosis, proliferation and differentiation of B cells and B-cell malignancies. Strong up-regulation of KLF4 was detected in MM cell lines in the process of apoptosis suggesting a role of KLF4 in MM progression [105]. Nestin is a remarkable protein that is found in rapidly dividing cells of developing and regenerating tissues [109]. Therefore, it is surprising that this gene and protein expression of the stem cell marker nestin has been detected in terminally differentiated PCs of MM patients [83, 110]. Moreover, our recent work proved that nestin protein is a tumor specific marker for $CD138^+38^+$PC of MM [110]. Expression of nestin, a marker of stem/progenitor cells in malignant PCs that are considered to be terminally differentiated, is highly controversial and indicates that nestin might play an exceptional role in the pathology of MM. However, biological or clinical implications of nestin have not been determined in monoclonal gammopathies or other hematological malignancies so far.

Summarizing these findings supports the hypothesis that stem cell-like features are not rare in monoclonal gammopathies and might indicate the existence of inducible stem cell properties in more differentiated cells than was initially thought [27, 111] (see **Fig. 2**).

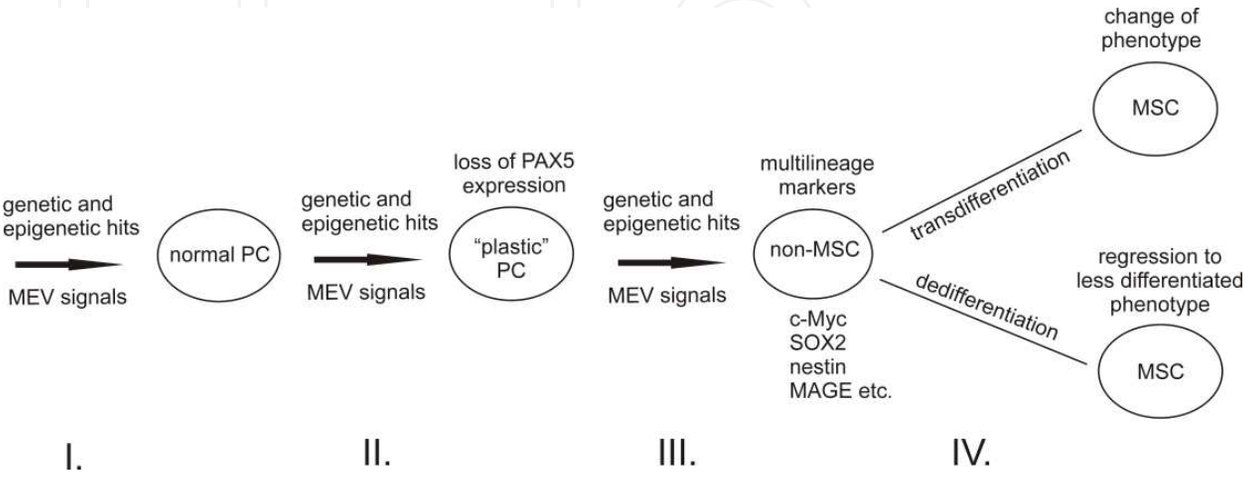

Figure 2. Model of inducible stem cell properties in myeloma

5. Putative role of nestin in myeloma plasticity

Neural stem cell marker nestin was first identified in neuroepithelial stem/progenitor cells of the rat central nervous system (CNS) by Hockfield and McKay in 1985 [112]. Nestin is detected in a wide range of undifferentiated tissues under normal and pathological conditions [113-116]. Expression of nestin is a common feature of multipotent proliferative progenitor cells with self-renewal and regeneration potentials. During terminal differentiation, nestin expression is silenced but can be reactivated upon injury or other pathological conditions such neoplastic transformation. The human nestin gene is located at 1q23 locus and is composed of four exons and three introns. Nestin expression is driven by a minimal promoter that is activated by transcriptional factor Sp1 [117]. Moreover, epigenetic regulation was also demonstrated. Results indicated that histone acetylation might be sufficient to mediate activation of nestin transcription [118].

Nestin protein belongs to a large family of intermediate filament (IF) proteins that are encoded by more than 70 genes expressed in a time and site-specific manner in metazoan cells. Members of the IF family are divided into six classes of proteins according to their structure, properties and localization [119]. Nestin, a class VI protein, is characterized by a α-helical central 'rod' domain which is typical for all IF, that contains repeated hydrophobic heptad motifs, a short N-terminus (head) and a very long C-terminus (tail) (**Fig. 3**) C-terminus is suggested to function as a linker or cross-bridge between intermediate filaments, microfilaments and

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microtubules [120]. The molecular weight of human nestin protein is ~ 200 – 220 kDa; more often it is found in its glycosylated form with molecular weight of 240kDa [121, 122]. Nestin does not fold by itself most likely because of its very short N-terminus. Therefore, nestin requires the presence of other IF proteins such as type III vimentin, desmin or type IV α-internexin [109, 123, 124].

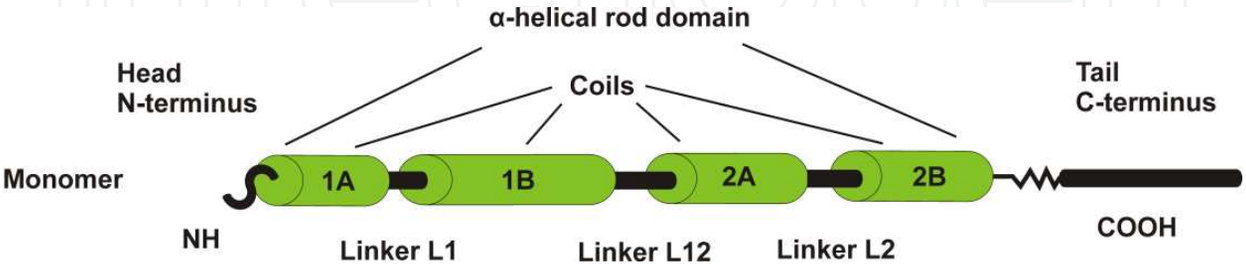

Figure 3. Structure of intermediate filaments (adopted and modified from Michalzyk & Ziman, [2005]

5.1. Cellular roles of nestin

An important regulator of nestin organization and dynamics during mitosis is cdc2-mediated phosphorylation. Phosphorylation/dephosphorylation of nestin may modulate disassembly and assembly of intermediate filaments [125]. These processes might play a role during increased cytoplasmic trafficking in progenitor cells undergoing division or in migrating interphase cells [126-128]. Nestin was shown to participate in asymmetric redistribution of cytoskeletal proteins and other factors to daughter neuroepithelial cells [129]. Nestin structure serves also as a scaffold for cdk5/p35 activity resulting in a cytoprotective effect against stress induced cell death in neural progenitor cells [122, 130]. Moreover, nestin is supposed to be a major determinant in suppression of anti-proliferative activity of glucocorticoid receptors (GR) in undifferentiated tissues by anchoring GRs in cytoplasm but cells lacking nestin accumulate GRs in the nucleus [131]. This mechanism might be responsible for glucocorticoid resistance seen in many cancers.

5.2. Nestin in multiple myeloma

Biological or clinical implications of nestin have not been systematically studied in monoclonal gammopathies or other hematological malignancies so far. The first mention of nestin expression in MM was described in malignant PCs of 5 MM patients and two myeloma cell lines (NOP2 and Liu01) by Liu et al. (2007). Authors referred to the existence of $CD56^+$ primary MM cells expressing neuronal markers, such as nestin, neuron-specific enolase and β-tubulin III [83]. Expression of nestin was reported to be stimulated by the Notch signaling pathway in human gliomas [132]. The Notch pathway plays also an important role in survival and proliferation of malignant PCs and negatively affects bone disease in MM [133]. Our recent

work proved that nestin protein is a tumor-specific marker for $CD138^{+}38^{+}$PC of MM patients [110]. Regarding the fact that myeloma PCs appear to be mature, terminally differentiated cells with a low proliferative potential, the presence of nestin is very surprising. Cells expressing nestin may represent a transient population undergoing dedifferentiation into CSC-like phenotype. Providing that PCs possess a truly dedifferentiation potential, the direction of dedifferentiate needs to be elucidated and whether or not this process is reversible.

Unexpectedly, our results showed that nestin was present in one-third of cases in 50% to almost 100% of PCs. Also expression of MAGE genes was found in majority of PCs [102, 134]. Thus, if nestin is expressed in most cells, it means that majority of PCs might gain stem cell features and could repopulate the tumor after therapy. These findings take into question the hierarchical model of myelomagenesis based on the presumption that MM originates from a minor population of MSC. Conversely, stochastic clonal evolution model better describes mechanisms responsible for recurrence of MM. This model suggests that majority of tumor cells have a character of stem cells and may repopulate tumor cells after treatment [135]. Plasticity of PCs better fits the clonal evolution model because weakly proliferating PCs would not be able to quickly repopulate the tumor, therefore we hypothesized that they need to convert into more rapidly dividing cells.

Association of nestin with plasticity of terminally differentiated cell types were demonstrated in the study of metaplastic conversion of mature pancreatic epithelial cells [136] Metaplastic conversion is defined as the replacement of one differentiated cell type by another mature cell type and is frequently associated with an increased risk of subsequent neoplasia. Causes of these changes remains unclear but one possible mechanism may be transdifferantiation of one mature cell type to another one either directly or via an undifferentiated transient cells (rev. in [137]). Means *et al.* (2005) showed that mature acinar cells can convert into ductal epithelia under EGFR signaling. Metaplastic changes were accompanied by occurrence of nestin-positive intermediates similar to nestin-positive precursors observed during early pancreatic development. Results of this study proved a real trans-differentiation potential of mature mammalian cells and indicated that plasticity of mature cell types may play a role in the generation of neoplastic precursors. Whether nestin-positive myeloma PCs might represent particular intermediates undergoing changes leading to the occurrence of cells with a changed (transdifferantiation) or less differentiated phenotype (dedifferentiation), it remains a matter for future research. On the other hand, nestin expression might represent only a byproduct of tumor transformation without any association with myeloma heterogeneity. However, the dynamic character of nestin network plays an important role in the key cell processes such as proliferation, migration and cell survival. Nestin polymerization/ depolymerization influences intracellular signaling, it is likely responsible for rapid redistribution of intracellular proteins, cytoskeletal remodeling and/or might function as a scaffold for protein interactions [122, 125, 129, 130] (**Fig. 4**). All these properties might represent an important prerequisite for myeloma plasticity.

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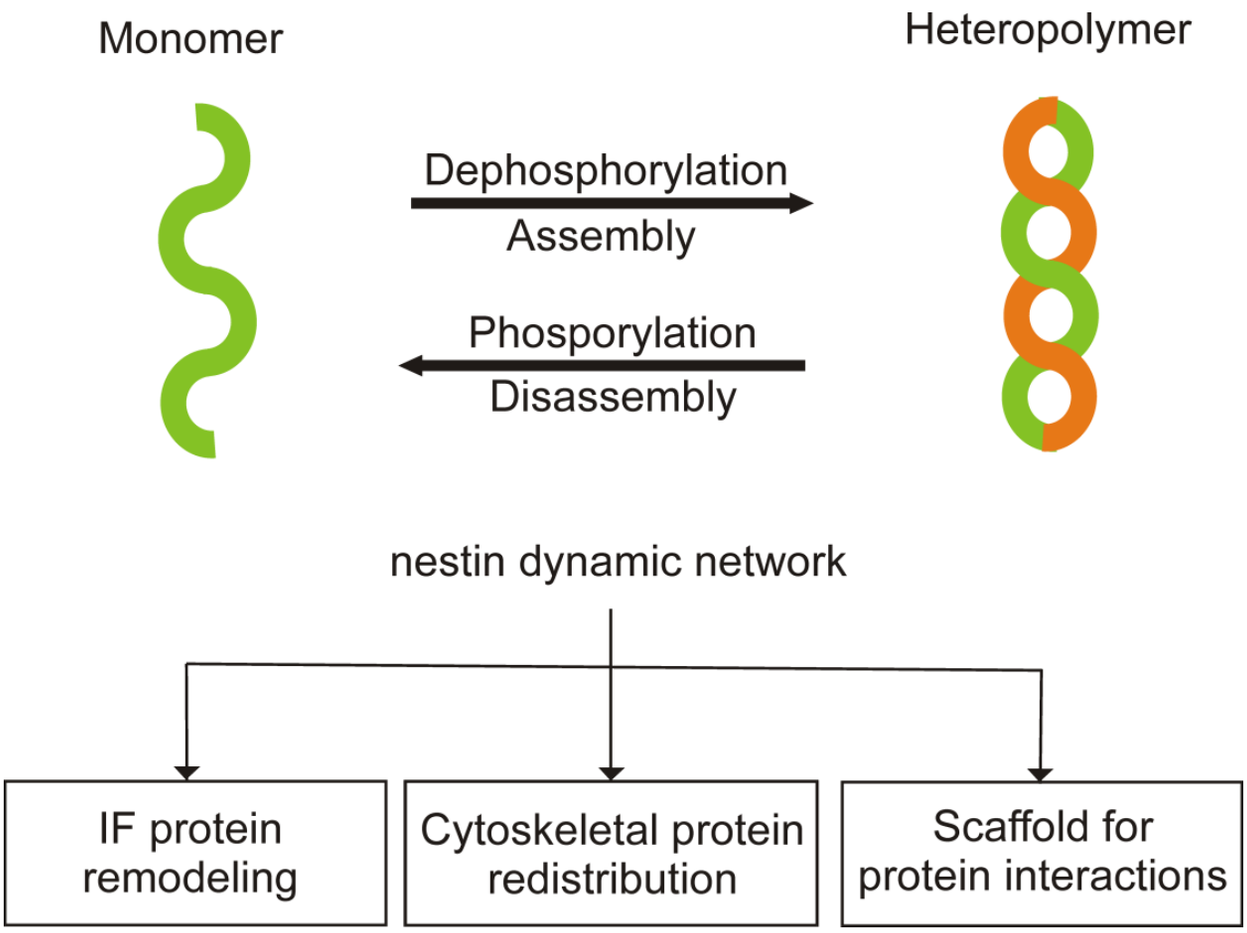

Figure 4. Nestin remodeling and cellular functions

6. Cancer stem cell phenotype and tumor microenvironment

Recent knowledge supports the hypothesis that altered BM microenvironments participate in both mechanisms leading to tumor progression; induction of stem cell features and stimulation of angiogenesis. Cancer stem cell phenotypes may be a plastic state induced in cancer cells depending upon microenvironmental signals, such as hypoxia. Hypoxia has a great impact on the production of angiogenic factors but it is also a crucial regulator of the stem cell phenotype. Several reports have shown that hypoxia and HIFs are involved in maintaining a stem-like state in normal tissues [138]. One example can represent hematopoietic stem cells that reside in regions regulated by oxygen tension. It is hypothesized that undifferentiated phenotypes of these cells relies on HIF activity in hypoxic areas. Hypoxic areas in tumors might be an analog to stem cell niches in normal tissues. Furthermore, Yoshida *et al.* (2009) have shown that hypoxic conditions significantly improve generation of iPSC [139]. Growing knowledge of cancer stem cell biology suggests that hypoxia may act as a critical regulator of the cancer stem cell phenotype.

Series of experiments have demonstrated that hypoxia is responsible for altering the cellular phenotype by causing an increase in proliferation, self-renewal and upregulation of stem cell genes in both CSC and non-CSC. Several groups have shown that hypoxia can regulate histone methylation and thus alter the epigenetic status of cancer cells [140-142]. Tumor hypoxia also correlates with poor outcome of patients. HIFs were shown to induce the embryonal stem cell-like transcriptional program, including *OCT4, NANOG, SOX2, KLF4, MYC*, and micro-RNA-302 in cancer cell lines of prostate, brain, kidney, cervix, lung, colon, liver and breast tumors [143]. Hypoxic microenvironment potentiates biological effect of Notch signaling in adenocarcinoma of the lung or alters gene expression of neuroblastoma cells to induce more immature phenotype [144, 145]. CD133, a cancer stem cell marker, has been reported by several groups to be upregulated under hypoxic conditions [146, 147]. McCord et al. [2009] showed that hypoxia not only increased the sub-population of glioblastoma cells positive for CD133, but also enhanced expression of other stem cell markers, such as SOX2, OCT4 and nestin [148]. Low oxygen levels induced also HIF-2α expression that can increase the expression of stem cell-associated genes and confer tumorigenic potential to non-CSC [140].

The BM microenvironment of MM is also hypoxic, and myeloma PCs are long term exposed to low oxygen levels. Tumor adaptation to hypoxia is mediated by the production of HIF-1 [149]. Both HIF-1α and HIF-2α have been reported to be activated in MM patients resulting in stimulation of angiogenesis [150]. Although a role of BM microenvironment is generally recognized as a crucial factor affecting myeloma development and support progression, it is surprising that an importance of hypoxic microenvironment for modulation of plasma cell phenotype have never been studied in MM.

7. Conclusion

Despite the achievements of currently used therapy, MM remains difficult to treat. Novel agents such as inhibitors of proteasome or immunomodulatory drugs have prolonged survival of patients with MM and even some patients persist in long term remission. However, there is a little known about mechanisms of myeloma development or the population responsible for the origin and relapse of the disease. Many scientists have tried to explain causes of the relapse but none of their theories have been conclusively confirmed so far. In this review, we explain inconsistencies among particular concepts and the inability to detect cells of origin by the plasticity potential of myeloma PCs. Plasticity of myeloma PCs might be a cause of vast phenotypic heterogeneity of MM and different characteristics of putative myeloma precursors. Under specific signals from aberrant microenvironments, PCs might undergo dedifferentiation/transdifferentiation changing their phenotype profile, and acquire stem cell-like properties to ensure survival. Therefore, an effort to target the specific cell type based only on surface markers is not sufficient. Instead, it is necessary to concentrate on pathologic mechanisms responsible for the transition from non-CSC to CSC like cells. Additional focus on adjacent microenvironments and specific prevention of stem cell-like conversion might increase success of future therapy.

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